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14995

Cytoplasmic Inclusion Bodies in the Pancreas and Salivary Glands of Small Laboratory Animals.

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During the examination of microscopic sections of the viscera of hooded rats that had been used in other experiments, large cytoplasmic inclusion bodies were noted in the acinus cells of the pancreas and of the salivary glands of a large proportion of the animals. The presence of the inclusions in untreated control animals and the resemblance of the inclusions to those described in mice affected with the virus of infectious ectromelia[1] made it seem worth while to survey our own and other colonies of small experimental animals and to attempt to influence the incidence of the inclusions by the means at our disposal.

Materials and Methods. Small experimental animals of 3 separate laboratories (labeled B, C, and D in Table I) of the Faculty of Medicine of McGill University were examined in addition to animals housed in our own department (labeled A in the table). The 4 laboratories were located in 3 separate buildings and there was no direct contact between the several animal colonies. The animals examined were apparently healthy and all were killed by exsanguination or illuminating gas. Blocks of tissue were removed from all of the organs, preserved in formalin and his-

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[1] Marchal, J., *J. Path. and Bact.*, 1930, **33**, 718.

TABLE I.
Incidence of Inclusion Bodies in Pancreas and Salivary Glands.

Colony	Animal	No.	Age	Incidence of inclusions Pancreas, %	 Salivary glands, %
A	Hooded rats	120	2-20 mo.	75	12
	White ”	10	2 yrs.	80	10
	White mice	10	3-12 wks.	50	0
	Wild ” (trapped in animal quarters)	2	unknown	100	0
	Rabbits	3	”	66	0
	Guinea pigs	5	6-12 mo.	0	0
B	Hooded rats	15	3 wks.-6 mo.	47	20
	White mice	10	2 wks.-4 mo.	20	0
C	Hooded rats	10	adult	40	30
	Rabbits	3	unknown	66	0
D	Hooded rats	13	1-8 mo.	23	15

TABLE II.
Incidence of Inclusions in Hooded Rats Treated with Pilocarpine or Atropine.

Drug	No. of animals	Incidence of inclusions Pancreas, %	 Salivary glands, %
Pilocarpine nitrate (100 mg/kilo subcutaneously)	10	80	10
Atropine sulfate (60 mg/kilo intraperitoneally)	10	50	0
Untreated controls	5	80	20

tological sections stained with hematoxylin and eosin. All organs were examined microscopically until it became apparent that inclusions were present only rarely in sites other than the pancreas or salivary glands. The relative abundance of the inclusions in these latter organs was graded on a scale of zero to 4 plus.

Results. The results of the survey are set forth in Table I. A factor too cumbersome to be included in the table, but one which may be of importance is the date on which each animal was killed. A scatter graph constructed by plotting the relative abundance of inclusions in the pancreas of the hooded rats from our own laboratory against the date of examinations showed a definite peak of incidence and abundance of the inclusions in May and June for the 12 months embraced by the survey. The fact that no large number of animals was killed in April, September, or October made the peak less than absolute, but did not deny the marked variation in the months when large and comparable groups of animals were killed.

As shown in the table, the range of ages in each group is rather wide. When the animals whose ages were known exactly were studied individually, it was noted that no animal under 30 days old exhibited inclusions in either the pancreas or salivary glands, and that the oldest animals had the highest incidence. A litter of newborn hooded rats was sampled at weekly intervals, and no inclusion bodies appeared until the litter was exhausted at 8 weeks. In the last animal sacrificed, inclusion bodies were found in small numbers, but in the pancreas only.

The inclusion bodies (Fig. 1) as seen in the pancreas and in all 3 histological types of salivary gland tissue were found only in the acinus cells of these glands. Although frequently a single inclusion was seen in only one cell of an acinus, more often the inclusions appeared in clusters with from one to 3 bodies contained in each of 3 or 4 contiguous cells. The inclusions were cytoplasmic and generally located in that part

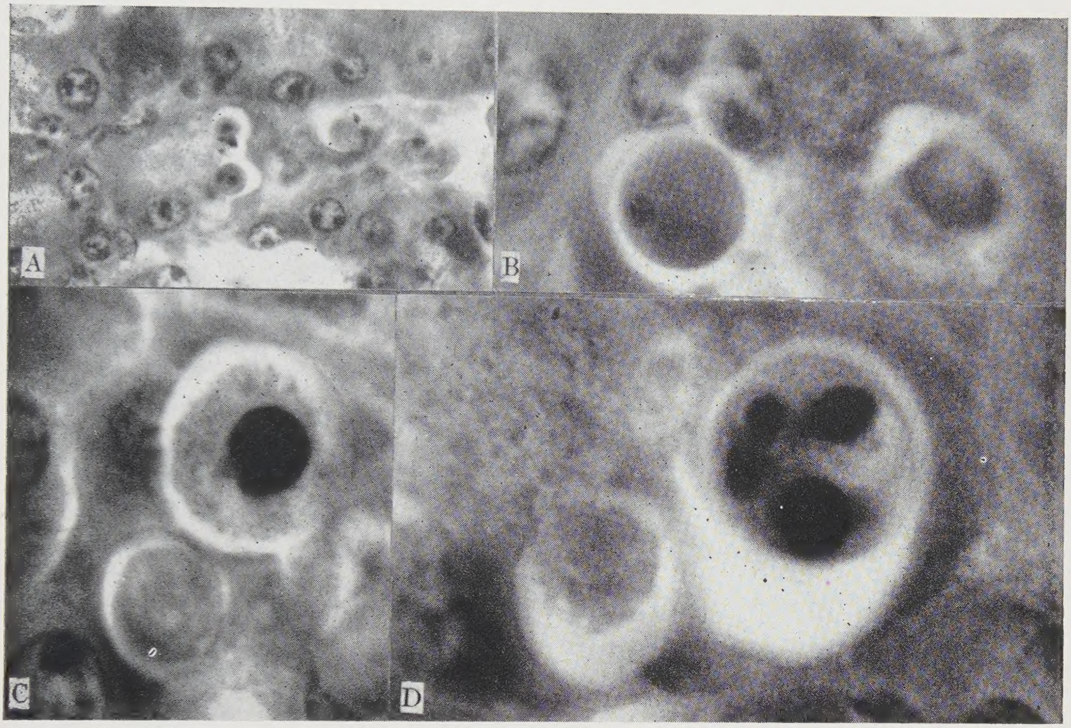

FIG. 1.

Photomicrographs of Cytoplasmic Inclusion Bodies in the Pancreas of Hooded Rats.

A. A cluster of inclusions in adjoining acini. Three large and several small inclusions may be seen. × 810.

B. One large hyaline appearing inclusion, clearly compressing the nucleus. Another inclusion to the right is slightly out of focus. × 2800.

C. A cluster of inclusions, one of which has a single basophilic granule. × 2800.

D. Two inclusion bodies, one of which contains 4 basophilic granules. The smaller inclusion consists of granules which resemble the zymogen granules visible in the left upper corner. × 3300.

of the cell nearest the lumen of the acinus. They were remarkably uniform in appearance in both pancreas and salivary glands in all of the animals regardless of species. They were spherical and large, often reaching a diameter of 15-20 micra, but in each animal varied widely in size. Their staining reaction was also variable, but tended in the main to be strongly eosinophilic. The largest inclusions usually contained from one to 5 small round basophilic granules. The eosinophilic part of the inclusions had either a homogeneous hyaline appearance or was finely granular. In some sections of the pancreas these granules strongly resembled the adjacent zymogen granules. In the salivary glands, the zymogen granules were slightly paler than those of the inclusions. Each of the inclusions was contained in an otherwise empty vacuole slightly larger than the inclusion body itself. The nuclei of the affected cells were pushed aside and were occasionally pyknotic, but showed no other sign of degeneration. Evidence of inflammatory response was lacking. Intranuclear inclusions were not seen, nor were inclusions of any kind found in the duct cells of either pancreas or salivary glands. It is noteworthy that the cytoplasmic inclusions in salivary glands were encountered only in rats.

Because facilities for virus research were not available, it was not possible to investigate by direct experiments the question of the possible virus etiology of the cytological changes described. The resemblance of the eosinophilic granules contained in some of the inclusions to the zymogen granules in neighboring cells suggested the possibility that the formation of the cytoplasmic bodies might depend on a secretory disturbance. If this were true it seemed possible that their incidence, abundance, or appearance could be influenced by pilocarpine or atropine. Accordingly, the effects of these drugs were studied.

Hooded rats of comparable ages were given pilocarpine or atropine and killed in pairs at hourly intervals for 5 hours after injection. The details and results of this experiment are contained in Table II. It is evident from the figures presented in the table that the injection of pilocarpine produced no difference in the incidence of the inclusions from that observed in the control animals, nor was there any difference in the abundance or appearance of the inclusions in the affected animals. The incidence of the inclusion bodies was distinctly lower in the animals treated with atropine than in the controls, but it is noteworthy that there was no decrease in the abundance of the inclusions in the animals that presented them nor any change in their microscopical appearance. It is questionable, therefore, whether the decrease in incidence is really significant.

Discussion. Histological sections of pancreatic tissue containing typical inclusions were sent to Professor E. V. Cowdry for his opinion. He was kind enough to comment that he had never before seen similar cytoplasmic bodies in the pancreas. He noted the fact that there was no evidence of destruction of the affected cells and no sign of any inflammatory reaction, and expressed the opinion that a virus etiology was very questionable. Pointing to the similarity between the granules in the inclusions and the zymogen granules in neighboring cells, he proposed that the inclusions might represent some sort of temporary secretory retention and suggested trying the effects of pilocarpine and atropine. The essentially negative results of the experiments with these drugs offer no support for the idea that the inclusions were caused by a disturbance of secretory function, but neither do they completely exclude this possibility.

The possibility of a dietary factor being responsible for the formation of the inclusion bodies was considered. All of the rats examined, except for those from Laboratory D, were fed with Purina fox chow, which is commonly employed as the standard diet for small laboratory animals. This diet was supplemented with whole wheat bread in two laboratories. In Laboratory D, half the rats were raised on another brand of chow, and their growth was believed to be slower than usual. Among these latter animals inclusions were found more frequently than in other animals from the same laboratory, but the incidence in this sub-group still was lower (33% in the pancreas) than that in the other colonies. The histological criteria of avitaminoses (*i.e.*, epithelial metaplasia, muscle degeneration, etc.) were lacking in these and all the other animals. These facts offer no evidence to indicate that any dietary deficiency was a factor.

Search of the literature of the last 20 years failed to reveal any mention of the occurrence of inclusions similar in morphology and distribution to those described above. The closest resemblance to the lesions in the pancreas is shown by the cytoplasmic inclusion bodies described in this organ in association with infectious ectromelia of mice.[1] In this spontaneous, and often fatal, virus disease, similar, but smaller, inclusions are also found in the skin, intestinal glands, and salivary glands. A spontaneous disease comparable to infectious ectromelia of mice is unknown in rats, but inapparent ectromelia has been induced in rats by Reames[2] and by Burnet and Lush[3] by nasal inoculation. The latter investigators were able to demonstrate inclusion bodies in the nasal mucous membranes of some of the rats, but no inclusions were described in other tissues.

Pearson[4] and Cowdry,[5] among others, reported the presence of cytoplasmic inclusions in the duct cells of the salivary glands in animals affected by the salivary gland virus, in association with the more typical intranuclear inclusions. The cytoplasmic bodies, however, are smaller than those we have seen, are basophilic, and do not contain granules. In Pearson's cases, the cytoplasmic inclusions were seen in the acinus cells of the salivary gland occasionally, but most often in the duct cells, where the nuclear inclusions are characteristically present. McCordock and Smith[6] reported visceral inclusions in mice affected with the salivary virus. The inclusions were present in the acinar cells of the pancreas, as well as in other organs, but were chiefly intranuclear in type, although occasional basophilic cytoplasmic inclusions were found. From consideration of these facts, it is apparent that there is no morphological evidence of relationship between the inclusions we have seen and the salivary virus disease.

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[2] Reames, Harold R., *J. Infect. Dis.*, 1940, **66**, 254.

[3] Burnet, F. M., and Lush, Dora, *J. Path. and Bact.*, 1936, **42**, 469.

[4] Pearson, E. F., *Am. J. Path.*, 1930, **6**, 261.

[5] Cowdry, E. V., and Scott, G. H., *Am. J. Path.*, 1935, **11**, 647.

Inclusion bodies similar to those found in our animals were seen in the livers of 3 mice by Campbell[7] who thought they resembled the inclusions of ectromelia, but he was unable to prove their etiology. Shortt and Lahiri[8] described bodies which they called "eosinophilic vacuole-like structures" in the serous acini of the salivary glands of dogs affected by rabies. Later investigation showed similar inclusions to be present in non-rabid animals. These workers considered the bodies they observed to be identical with ectromelia inclusions, but were convinced that the inclusions in their animals resulted from hypersecretion. They attempted to prove this by showing a vast number of inclusions in dogs given pilocarpine, but unfortunately, the salivary glands were not biopsied before the drug was given, and their results are therefore inconclusive. Rosin and Doljanski[9] reported the presence of erythrocytes in the cytoplasm and nuclei of liver cells in rats given urethane and allyl formate. In late stages of degeneration, the red cell aggregates in the cytoplasm resembled the inclusion bodies seen in our animals, but there is no reason to believe that they were similarly formed, since hemorrhage was prominent in their animals and absent in ours.

Summary. Hitherto undescribed cytoplasmic inclusion bodies have been found in the pancreas of apparently healthy mice, rats, and rabbits and in the salivary glands of the rats. In both organs the inclusions were confined to the acinus cells. They were not encountered in guinea pigs. Their etiology is unknown. The possibility that the inclusions were caused by disturbance of secretory function was not supported by the results of experiments to test the effects of pilocarpine and atropine. Pilocarpine provoked no change in the incidence, abundance, or appearance of the inclusions and atropine, if it had any effect at all, was only mildly inhibitory. There is no evidence that dietary factors were responsible. Evidence for viral origin is only suggestive and consists of the following points: 1. The presence of inclusion bodies in the absence of nonviral factors known to produce them. 2. Strong resemblance of the inclusions to those described in infectious ectromelia of mice, a disease of proved virus etiology. 3. Absence of inclusions in animals under 30 days of age and an increasing incidence thereafter with increasing age. 4. A seasonal variation in the incidence and abundance of the inclusion bodies.

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[6] McCordock, H. A., and Smith, M. G., *J. Exp. Med.*, 1936, **63**, 303.

[7] Campbell, J. A., *J. Path. and Bact.*, 1939, **48**, 223.

[8] Shortt, H. E., and Lahiri, B. N., *Indian J. Med. Res.*, 1934, **21**, 587.

[9] Rosin, A., and Doljanski, L., *Brit. J. Exp. Path.*, 1944, **25**, 111.

14996

Induced Resistance in Inbred Homozygous Rats to a Lymphosarcoma Autogenous to the Strain*

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In previous studies with mouse sarcoma 180[1] it was found possible to induce a resistant state in hybrid mice by implants attenuated by specific doses of radiation. The results of these studies may be briefly summarized as follows:

A dose of about 60,000 r in air was found to be required to prevent the proliferation of the sarcoma cells in tissue culture, *i.e., in vitro.* Later it was also shown that this dose was destructive for their immunizing action *in vivo.* Implants of the same type of tumor irradiated with doses of about 5,000 r in air, although failing to produce a detectable tumor *in situ,* nevertheless rendered the animal immune to further viable implants. Tumor grafts irradiated with doses smaller than about 5,000 r in air produced tumors following inoculation.

The present study was concerned with the question as to whether or not this phenomenon can be applied to tumors of a known genetic origin, grown in an inbred strain of animals. The importance of this question is realized when one considers the problem of inducing resistance to spontaneous tumors. Accordingly, animals of an inbred strain were used and a type of tumor which originated spontaneously in the same strain. These were obtained from Dr. Halsey J. Bagg with the following data:

The rats were bred by continuous brother and sister mating for more than 15 years and hence could be considered homozygous. The tumor, which originated in this strain of rats, and had been diagnosed by the late Dr. James Ewing as a reticulum cell type lymphosarcoma, had reached the 75th generation of transfer at the start of this experiment. The tumor takes were practically 100% in young rats. Spontaneous regression very rarely occurred among these animals.

Experimental. A rat bearing a 10-day-old lymphosarcoma at its 75th generation of transfer and 8 normal male rats 6 weeks old were supplied by Dr. H. J. Bagg at the start of these experiments. The tumor was removed from the rat under strictly aseptic conditions and cut into small fragments with sharp scissors. Six male rats were implanted by means of a trocar, each receiving one tumor fragment placed under the right axilla. All 6 rats developed tumors 8 to 10 days following implantation. The tumors grew progressively during the next 8 days, thus establishing 100% takes and progressive growth of the tumor in a new environment. Later quantities of rats were periodically supplied by Dr. Bagg. Then the experiment proper was started.

The technical procedure used here was the same as in the previous experiments with mouse sarcoma 180[1] of which a concise summary will be given here.

For each experiment a 10- to 12-day-old tumor, removed from the rat under strictly aseptic conditions was used. This was cut into small fragments with very sharp scissors. Portions of the tumor fragments were spread on a No. 1 round coverslip which had previously been attached to a square mica sheet, and were covered with a Maximow slide and sealed with paraffin. To avoid any evaporation of the tumor-tissue water, a fragment of moist filter paper was placed in the concavity of the Maximow slide. Physical factors for irradiation were: 200 KV, 20 ma, 0.5 mm Cu, 1.0 mm. Al and a H.V.L. equivalent to 0.9 mm Cu. Immediately after irradiation the tumor fragments were implanted into a certain

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[1] Goldfeder, Anna, *Radiology,* 1942, **39**, 426.

TABLE I.
Effects of Filtered X-rays on Implants of Bagg's Reticulum Cell Type Lymphosarcoma (200 KV 20 ma ½ mm Cu + 1 mm Al. H.V.L. equal to 0.9 mm Cu).

Exp. No.	r in air*	Takes, %	Latent period, days	Initial tumor size, mm	Max. tumor size, mm	Remarks†
1	500	100	8	9x9x6	32x28x19	
2	1000	100	8-10	8x8x5	29x26x17	Max. size of tumor designates size tumor reached 2 weeks after first measurement.
3	1500	100	9-10	7x6x4	18x16x12	
4	2000	100	14	7x4x4	16x12x8	With increase of X-ray dose applied to implants, an increase of latent period and a decrease of max. tumor size occurred.
5	2500	60	18	6x6x4	12x9x6	
6	3000	0	0	0	0	

* r in air—roentgen units in air.
† Each experiment consisted of 6 to 8 rats.

TABLE II.
Resistance Induced in Homozygous Rats to a Lymphosarcoma Autogenous to the Strain.

Exp. No.	r in air*	No. of implanted rats	No. of takes	No. of re-implanted rats	No. of resistant rats	Remarks
1	2200	10	7	3	3	One of re-implanted rats produced a tumor which regressed after having reached a size of 12x10x7 mm. This rat became likewise immune.
2	2300	8	6	2	2	
3	2400	8	6	2	2	
4	2500	8	5	3	3	One of re-implanted rats produced a tumor which regressed after having reached a size of 15x19x8 mm. This rat became also immune.
5	2600	8	2	6	5	Two of the re-implanted rats produced tumors which eventually regressed, rendering the animals immune.
6	2300	8	5	3	3	

* r in air—roentgen units in air.

number of rats by means of a trocar. Only one tumor graft was implanted into each rat. Since the bore of the trocar was about 2.0 mm, the tumor grafts were approximately of the same size for each rat. The time elapsing from the removal of the tumor from the animal and implantation of the irradiated tumor fragments was about one hour. The same technical procedure was used for control animals except that these were implanted with non-irradiated tumor grafts.

Since the radiosensitivity of this type of tumor was not known, the first task was to determine the lethal and sublethal doses for the proliferation of tumor-grafts *in situ*. The same criterion for radiation effects were used here as in previous experiments,[1] namely: The latent period (which designates the time elapsing between the implantation of the tumor fragments and the first occurrence of a detectable tumor in the animal); the percentages of takes and size of tumor, initial and maximum. The time of 2 weeks elapsing from the first measurements of the tumors was arbitrarily designated the maximum size. This was done because the lymphosarcoma type of tumor is fast growing and after 2 weeks proliferation in the animal, it starts to break through the skin.

Results. In Table I are recorded the effects of filtered X-rays on implants of the reticulum cell type lymphosarcoma. Since the first task was to determine the approximate sublethal, and lethal dose of irradiation, the exposures were given within ranges of 500 r in air. As can be noted in this table up to a dose of 2,000 r in air applied to the implants resulted in 100% of takes. The effects of irradiation started to be apparent with a dose of 2,000 r in air. Although 100% of takes were still produced by implants exposed to the above mentioned dose, however, the latent period was extended to 14 days instead of 8 to 10 days as occurs among control animals.

With the increase of the X-ray dose applied to the implants a sudden decrease in percentages of takes occurred with a corresponding increase of the latent period. Thus a dose of 2,500 r in air resulted in 60% of takes with a latent period of 18 days, while implants exposed to 3,000 r in air failed to produce a detectable tumor in the animals. From the results recorded in Table I it seems reasonable to assume that the threshold dose for this type of tumor would be between 2,200 and 2,600 r in air. Further experiments carried out within the above range of roentgens are recorded in Table II.

An inspection of Table II reveals the following: In Experiment 1 with a dose of 2,200 r in air 7 out of 10 rats developed tumors. The remaining 3 which failed to produce tumors after a period of 28 days were reimplanted with freshly excised viable tumor grafts. One of the 3 reimplanted rats produced a tumor which eventually regressed after having reached a size of 12x8x4 mm in diameter. This rat became likewise immune. All 3 rats are still alive and proving to be resistant to the reticulum cell type lymphosarcoma.

Experiments 2 and 3 gave similar results. Two rats of each experiment (of which the implants of Experiment 2 were exposed to 2,300 r in air and the implants of Experiment 3 were exposed to 2,400 r in air) failed to produce tumors, became refractory to further re-inoculations of viable tumor grafts.

In Experiment 4 of 8 rats which were implanted with tumor grafts exposed to 2,500 r in air 5 produced tumors. The remaining 3 rats were reimplanted. Only one of the reimplanted rats produced a tumor which later regressed after having reached a size of 15x10x8 mm in diameter. This rat became likewise resistant to fresh viable tumor grafts. All 3 rats are still alive and proving to be immune to the reticulo-cell type lymphosarcoma.

In Experiment 5 with a dose of 2,600 r in air, 2 out of 8 rats produced tumors. The remaining 6 rats were reimplanted with fresh viable tumor grafts 27 days following the first implantation of the irradiated tumor grafts. Three out of the 6 reimplanted rats produced tumors within 15 days following reimplantation of fresh viable tumor grafts. In 2 of these rats the tumors later regressed rendering both refractory to further viable tumor implants. Thus Experiment 5 resulted in 5 resistant rats.

In order to test the reproducibility of these results, an experiment with a dose of 2,300 r in air was repeated as shown in Experiment 6, Table II. In this case 3 of 8 rats became resistant to further viable tumor grafts.

The question is apparent why in each experiment only a certain percentage of animals could be made resistant although they were all implanted with tumor grafts attenuated with the same dose. This question may be explained by the following: not each tumor graft contained cells of a uniform viability. It is known for example that sensitivity of cells to a given dose of irradiation varies with the stage of mitotic activity; on the other hand, it may be assumed that the amount of implanted tumor grafts was insufficient to produce a resistant state in the animal. This assumption is supported by the fact that rats in which the tumor grew and later regressed, all became refractory to further tumor grafts. Recent tests not included in the table on 4 rats which became refractory to further viable tumor grafts 7 months ago revealed a persistent resistance. Thus, from these experiments the possibility of inducing a resistant

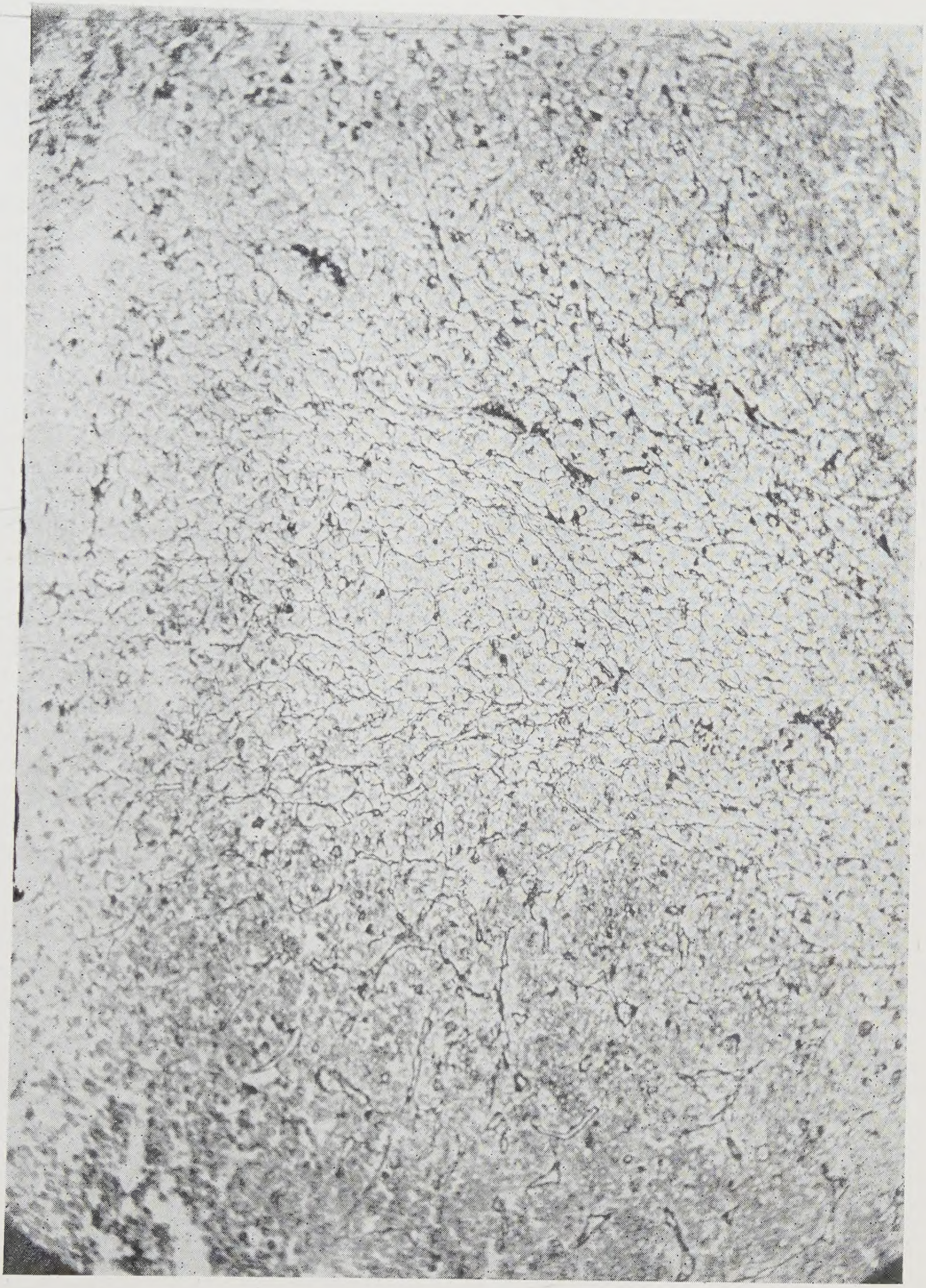

Fig. 1.
Reticulum cell type lymphosarcoma of rat. Silver stained. × 153.

state in a pure line of rats to autogenous type of tumor has been demonstrated.

It was regarded unnecessary to include a separate table of the records of the control animals, because of the fact that all the experiments gave 100% takes. In these animals the latent period varied from 8 to 10 days.

Further experimentation with a larger number of rats is in progress. A feature of this is the testing of rats which were successfully treated with X-rays on their possible immunity.

Discussion. An abundant literature has accumulated on the problem of a possible immunity to malignant growth since the classic observations of Clowes and Baeslack[2] on the existence of tumor resistance. These investigators observed that mice in which an actively growing tumor regressed spontaneously, or in which a transplantable tumor failed to "take" in the first instance, became resistant to malignant growth. However, the results obtained by subsequent investigators in pursuance of this phenomenon were confusing and contradictory.

Attempts were made to induce immunity in animals by a procedure of intracutaneous injection of tumor material. Some investigators[3,4] found this method effective, while others[5,6] failed to obtain positive results. The method of intracutaneous injection found a wide application in the work of Besredka, Gross *et al.*[7,8] These investigators reported that intracutaneous injection of Brown-Pearce carcinoma into rabbits rendered the animals, following regression of the tumors, immune to subsequent inoculation of the same type of tumor. These animals showed also to be refractory against intratesticular tumor transplants. Similar observations were made by the use of this method with Rous chicken sarcoma. However, the results obtained by Besredka *et al.* have not been generally substantiated. Several investigators[9,10,11,12] failed to produce immunity against Brown-Pearce carcinoma by the use of the intracutaneous method. On the other hand, several reports by various investigators substantiated the findings of Besredka *et al.*[13,14,15]

It is generally thought that the use of hybrid animals and transplantable tumors of unknown genetic origin might be responsible for the lack of uniformity among the results of the multitude of reported investigations. Space does not permit a comprehensive review of this problem. (The reader is referred to the bibliography.[16,17]) However, there are a few pertinent facts which are worthy of discussion at this point, and to which the results of this paper are relevant.

The viewpoint that the use of genetically unknown material was responsible for the contradictory results has received support in recent literature.[18,19] In these investigations it was shown that the induced resistant state bears a genetic relation to the type of living cells used and to the host. These authors used normal tissue cells, such as blood or embryo skin as the immunizing agent. Negative results as regards immunization against tumor growths were obtained in most instances. Inasmuch as the immunizing material used was derived from a pure line of animals, it was believed not possible to induce resistance in a pure strain of animals against tumors originating within their own strain. However, the experiments reported in this paper demonstrate the possibility of inducing such resistance in a genetically pure strain of rats.

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These divergent results raise the question as to whether the *type* of immunizing material used is not a more critical factor. It may be mentioned here that the author has been working, also, with another type of tumor, a mouse mammary adenocarcinoma which arose spontaneously in C57 "Y" black mice (also a pure strain of Dr. Bagg) in which, thus far, no immunity has been produced. It is interesting to note that the investigators[18,19] also used an adenocarcinoma. Consequently, we are faced with the possible phenomenon that immunity is possible in a pure line of animals when a reticulum cell type lymphosarcoma is used, whereas no such resistance can be produced with an adenocarcinoma.

Another experiment[20] has been reported in which immunity was successfully induced in a pure strain of C3H mice. Herein a mouse sarcoma produced by methylcholanthrene was used as the immunizing agent. A question may be raised as to whether a tumor produced by an extrinsic factor (chemical carcinogen) may be classified with spontaneous tumors in this respect. If so, then this work lends further amplification to the idea that the type of tissue used for immunization is a significant factor.

It is possible that this idea may have some bearing on the relative recurrences of different types of human malignancies. It is well known that certain cases, which either have had spontaneous regressions or have been successfully treated, suffer no further recurrences, whereas many cases, following apparently successful treatment, develop further malignant growth in later years. It is not unreasonable to infer that in the former cases the particular type of tumor suffered was capable of inducing a self immunity.

It is obvious, however, that much more and extensive work is necessary to resolve the question of immunity to cancer, particularly to spontaneous tumors. The method herewith described for inducing immunity appears to offer a good means of standardizing work along this line. If the thesis discussed here, that tumors may be biologically classified into those which can and those which cannot induce immunity, is ultimately to be validated, such standardization is necessary. Attempts have been previously made to induce immunity by implants attenuated by irradiation, for example, [21,22,23,24]. However, this work was done before the X-ray unit was quantitatively established and precluded accurately reproducible results. This obstacle has been overcome by the establishment of precise physical factors in the attenuating procedure and, consequently, as has been shown, results may be reproduced at will.

In general, it may be stated that standardization of procedures is a desideratum definitely to be hoped for, and which should greatly facilitate the solution of the cancer problem as a whole.

Summary. Rats of an inbred line, which were 100% susceptible to a reticulum cell type of lymphosarcoma arising spontaneously in the same strain, could, in an appreciable percentage of cases, be rendered resistant to the same tumor. The method consisted of implanting tumor grafts which were previously attenuated with specific doses of X-rays. In most cases the resistant state followed inoculation without the appearance of any visible tumor. In certain cases a tumor did appear but regressed after having reached a certain size. These likewise became immune. Hence this procedure eventually resulted in immunity to an autogenous tumor. Out of 58 control rats, only one spontaneous regression was observed. This rat likewise proved to be refractory to further viable tumor grafts of the same type.

The results of these experiments give evidence, apparently for the first time, that it is possible to render animals of a pure line immune against an autogenous tumor.

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[20] Gross, L., *Cancer Research*, 1943, **3**, 326.

[21] Haaland, M., *Proc. Royal Soc. London, Series B*, 1909-10, **82**, 293.

[22] Rohdenburg, G. L., *J. Cancer Research*, 1918, **3**, 181.

[23] Chambers, H., Scott, G. M., and Russ, S., *Lancet*, 1922, **1**, 212.

[24] Wood, F. C., and Prigosen, R. E., *J. Cancer Research*, 1925, **9**, 287.

14997

The Susceptibility of the Rodents, *Gerbillus gerbillus* and *Gerbillus pyramidum*, to Experimental Typhus Infection

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From the Cairo Unit of the United States of America Typhus Commission.

It is the purpose of this report to describe experiments which indicate the susceptibility of 2 rodents, *Gerbillus gerbillus* Olivier and *Gerbillus pyramidum* Geoffroy, to experimental typhus infection.† The rodents were supplied to the Cairo Unit of the United States of America Typhus Commission[1] through the courtesy of the Giza Ophthalmological Laboratory. They were trapped on the desert near Cairo, Egypt, where they occur in large numbers. Dr. Kamal Wassef, of the Department of Zoology, College of Science, Cairo, identified the two species.[2] The common Egyptian name of the species *G. gerbillus* is "far baiyoudeh"; of *G. pyramidum*, "far dimsi."

Preliminary tests with suspensions of infected lice taken from patients suffering from typhus fever showed that large numbers of rickettsiæ produced a fatal infection in gerbilles of both species by the intranasal and the intraperitoneal routes. The animals died 3 to 8 days after inoculation. Typical intracellular rickettsiæ were found in smears at autopsy. Further studies were made with the Breinl strain of classical louse-borne typhus in suspensions of infected yolk sacs.[3] It was found that the animals succumbed more regularly to smaller doses of rickettsiæ when the intravenous rather than the intraperitoneal route was used. The tail veins of both species are suitable for inoculation. When the gerbilles died several days after intravenous inoculation, smears of heart's blood occasionally contained a few large mononuclear cells with their cytoplasm entirely filled with densely packed rickettsiæ.

The smaller of the two species, *G. gerbillus*, was found to be more susceptible to fatal infection than the larger species, *G. pyramidum*, even when animals in the same weight range were selected for comparison (Table I).

The greater susceptibility of *G. gerbillus*, indicated by the experiment shown in Table I, was confirmed in another test in which the infective material was incubated for an hour at 37°C with normal rabbit serum for one group of gerbilles and with typhus immune rabbit serum for another. All of the gerbilles of both species survived the inoculum containing rickettsiæ and immune serum. Only 2 of a group of 12 gerbilles of the species *G. gerbillus* survived the inoculum containing rickettsiæ and normal serum, whereas, 6 of 7 *G. pyramidum* survived the same inoculum.

The specific protective effect of typhus

TABLE I.
Comparison of the Susceptibility of *Gerbillus gerbillus* and *Gerbillus pyramidum* to Experimental Typhus Infection by the Intraperitoneal and Intravenous Routes (Breinl strain of louse-borne typhus).*

Route of Inoculation	Concentration of infected yolk sac, %	Ratio of No. of survivors to total No. inoculated in each group	
		G. gerbillus	*G. pyramidum*
I.P.	20	1/4	3/4
	5	2/4	4/4
I.V	20	0/4	4/4
	5	1/4	3/4
	1¼	4/4	4/4

*All rats were in the 20-25 g weight range at the time the test was started. Each rat received 0.25 cc of the stated concentration of yolk sac suspension in saline, via tail vein. No anesthesia was used. The experiment was arbitrarily terminated at the end of 7 days.

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* Technical Expert of the National Health Administration of China.

† Gear and Davis have described the susceptibility of gerbilles of the closely related genus *Tatera* to experimental epidemic and murine typhus infection. See: Gear, J., and Davis, D. H. S., *Trans. Roy. Soc. Trop. Med. and Hyg.*, 1942, **36**, 1.

[1] Bayne-Jones, S., *Army Med. Bull.*, 1943, **68**, 4.

[2] Anderson, J., *Zoology of Egypt: Mammalia*, London, 1902, pp. 252, 255.

[3] Cox, H. R., *U. S. Pub. Health Rep.*, 1938, **53**, 2241.

immune serum was fully confirmed in several experiments with the species *G. gerbillus*. The sera used in the tests were taken from patients suffering from louse-borne typhus fever at various stages in their illness and in convalescence. Protective antibodies were demonstrated in the sera of patients toward the end of the febrile period and in one case as long as 3 years after the illness. Tests were not made at longer intervals. Protective antibodies were likewise demonstrated in some, but not all of the sera from persons who had received varying amounts of Breinl strain typhus vaccine (Cox type).

Both species were found to succumb to the Wilmington murine strain of typhus as well as to louse-borne typhus. Cells packed with rickettsiæ were found to be much more numerous in smears of peritoneal exudates made when the former strain was used.

The two infections produced a complete cross-immunity to one another. Gerbilles of both species, when tested 2 weeks to 2 months after a sub-lethal dose of either murine or louse-borne typhus, were immune to a dose of the other strain which killed all of the control non-immune animals in 3 to 7 days.

Relatively small amounts of killed rickettsial vaccine (louse-borne strain) administered subcutaneously in 2 doses one week apart, conferred immunity in gerbilles of the species *G. gerbillus* to at least one "certainly fatal dose" of a similar strain.

Specific complement fixing antibodies[4] were demonstrated in the blood of both species after inoculation with sub-lethal doses of louse-borne strains. The same result was obtained with the Wilmington murine strain. In the few tests which have been run thus far, there was very slight cross fixation between sera from gerbilles which had received either murine or louse-borne typhus. In some instances the complement fixing antibodies were observed several months after the infecting dose of typhus. The longest interval tested in these studies was 5 months and a positive result was obtained in that instance. Neither species was found to have developed positive Weil-Felix tests (proteus OX-19) as a result of infection with either louse-borne or murine typhus in the few tests completed at this time.

Since there has been a considerable amount of typhus fever in the region in which these rodents abound, the question has been entertained as to the possible incidence of typhus as a natural infection of the species. Inoculation of the brains of both species into guinea pigs and eggs in several trials has not disclosed evidence of typhus infection in either species, although an agent pathogenic for guinea pigs, possibly *Spirillum minus*, was encountered in the species *G. pyramidum*. The guinea pigs which had a febrile response after inoculation of gerbille brains did not develop complement-fixing antibodies to typhus, nor were they immune to challenge with known typhus strains. The sera of both species have been examined shortly after arrival in the laboratory and as yet no evidence has been obtained that the animals had previously suffered from typhus infection. When large doses of rickettsiæ were given, the control animals regularly died, and the gerbilles which had recovered from experimentally induced typhus had no evidence of sickness. This phenomenon, observed repeatedly, suggests that the two species in question either are not infected with typhus in nature, or if so, only rarely. The length of survival of rickettsiæ in the organs of gerbilles which have survived experimental typhus infection has not yet been determined.

The species *G. gerbillus*, in one or two instances, seemed to be slightly susceptible to the toxic effect of rickettsiæ.[5,6] Usually, however, the yolk sac suspensions employed in these studies did not contain a sufficiently large number of rickettsiæ to have had a very definite toxic effect.

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[4] Plotz, H., Wertman, K., and Bennett, B. L., from the Division of Virus and Rickettsial Diseases, Army Medical School, Army Medical Center, Washington, D.C., 1943, to be published.

[5] Gildemeister, E., and Haagen, E., *Deut. Med. Wchnschr.*, **66**, 878.

[6] Bengston, I. A., Topping, N. H., and Henderson, R. G., communication dated 11 July, 1942, Division of Infectious Diseases, National Institute of Health.

TABLE II.
Comparison of the Susceptibility of *Sigmodon hispidus hispidus** and *Gerbillus gerbillus*† to Experimental Typhus Infection.

Concentration of infected yolk sac (Breinl strain of louse-borne typhus) %	Ratio of No. of survivors to total No. inoculated in each group	
	S. hispidus	*G. gerbillus*
10	0/3	3/4
2.5	1/3	3/4
0.63	2/3	4/4
0.16	3/3	4/4

* The cotton rats were inoculated intracardially.

† The gerbilles were inoculated in the tail vein. Both species were inoculated with amounts which corresponded to their sizes: 0.1 cc of the stated yolk sac concentration for each 20 g of body weight.

In both species, a small rod-like form was frequently observed in the blood. It usually occurred in oval clusters approximately the size of a red blood cell. It stained red with Machiavello's stain. It was not found inside cells. Attempts to cultivate it in nutrient broth and in the yolk sac membrane of developing chick embryos were unsuccessful. However, its appearance was somewhat similar to that of typhus rickettsiæ, and the presence of the latter was only considered certain when smears showed the typical intracellular forms.

One experiment was conducted to compare the susceptibilities of *G. gerbillus* and the eastern cotton rat, *Sigmodon hispidus hispidus.*[7] The results shown in Table II indicate that *G. gerbillus* was slightly less susceptible to fatal infection than *Sigmodon hispidus hispidus.*

The species *G. gerbillus* proved to be useful for various purposes, particularly the demonstration of cross immunity between standard laboratory strains and newly isolated strains, the survival or death end point being superior to the interpretation of fever curves in guinea pigs.

Summary. *G. gerbillus* and *G. pyramidum,* two rodents which abound in Egypt, succumb to large doses of either murine or louse-borne typhus rickettsiæ by the intranasal, intravenous, or intraperitoneal routes. Protective antibodies in the sera of persons who have recovered from typhus fever and in the sera of persons who have been vaccinated against typhus fever have been demonstrated in tests with the species *G. gerbillus.* Both species were useful in various types of experiments in which survival or death was the end point. Neither species has yet been found to be infected with typhus under natural conditions. Specific complement-fixing antibodies were demonstrated in the sera of both species after inoculation with murine or louse-borne typhus. *G. gerbillus* was found to be slightly less susceptible to fatal infection than the Eastern cotton rat, *Sigmodon hispidus hispidus.*

The authors wish to thank the Giza Ophthalmologic Laboratory for supplying the gerbilles, Dr. Kamal Wassef for identifying the two species, the Egyptian Ministry of Agriculture for making available the fertile hens' eggs used in preparation of the infective material in the study, the Director of the Cairo Fever Hospital, Dr. Demerdash Bey, for facilitating the establishment of the United States of America Typhus Commission laboratory in his institution, and Dr. Andrewes of the National Institute of Health, Hampstead, England, for sending the cotton rats.

Sergeant Dworkowitz and Corporals Stearman, Hogan, and Cassell took part in this study and their assistance is gratefully acknowledged.

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[7] Snyder, J. C., and Anderson, C. R., *Science*, 1942, **95**, 23.

14998

The Susceptibility of the Rodents, *Gerbillus pyramidum* and *Gerbillus gerbillus*, to Experimental Tsutsugamushi Infection (Scrub Typhus)*

C. J. D. Zarafonetis. (Introduced by S. Bayne-Jones.)

From the Cairo Unit of the United States of America Typhus Commission.

The purpose of this report is to present experimental evidence which indicates the susceptibility of 2 rodents, *Gerbillus pyramidum* and *Gerbillus gerbillus*, to tsutsugamushi infection. The susceptibility of these animals to epidemic and murine typhus infection has been reported recently from this laboratory,[1] and the description, identification, source, and supply of the rodents used in these tests are the same.

Origin of tsutsugamushi strains used. Four strains of *R. orientalis*[†] were available for trial in these rodents. They are designated Imphal (= Imphal 8), Imphal 13, Calcutta, and Ceylon with the names indicating the locality in which they were isolated. Their derivations are as follows:

Imphal (= Imphal 8). Lt. Col. M. H. P. Sayers, R.A.M.C., injected 3 guinea pigs with a suspension of pooled blood clots which had been obtained from 5 patients with tsutsugamushi-like disease (on their 8th, 11th, 12th, 13th, and 20th days of disease, respectively). Rabbits were inoculated intra-ocularly with peritoneal washings from 2 of the 3 guinea pigs. The rabbits reacted typically and the strain was maintained in other rabbits for 7 generations, and then transferred to white mice. On January 7, 1944, 3 of the 3rd generation rabbits inoculated with this strain were received in Cairo, and on Feb. 19, 1944, infected mice of the 5th mouse passage were received. The latter line of this strain is named "Imphal 8."

Imphal 13. Capt. Parry, R.A.M.C., isolated this strain from blood taken from patients on the 4th and 7th days of disease by inoculating guinea pigs and then transferring to white mice and rabbits. Fourth generation mice of this strain were also received in February, 1944.

Calcutta. Major M. T. Parker, R.A.M.C., obtained this strain from the blood of a patient in his 12th day of disease. Washed blood clot material was inoculated intraocularly into a rabbit which showed iritis on the 11th day. One of the third passage rabbits was received in Cairo on January 7, 1944.

Ceylon. Major R. B. Lucas, R.A.M.C., isolated this strain and sent it to Dr. S. R. Savoor for purposes of identification. Dr. Savoor identified it as a strain of *R. orientalis* and sent infected mice of the 3rd generation to Cairo in June, 1944.

Passages of these strains in the Cairo laboratory. All 4 of these strains have been tested in gerbilles, and the passages carried out in the Cairo laboratory to the time of this writing are summarized as follows:

Imphal (= Imphal 8). The rabbit line of this strain was carried through 7 additional generations, making a total of 10 rabbit passages for it. Intra-ocular passages were made, and of the 26 animals used, half gave the characteristic response to the infection.[4] Passages to gerbilles were attempted from each rabbit generation except the 10th, by intraperitoneal inoculation of 0.1 cc of anterior chamber fluid from an infected rabbit's eye showing the characteristic involvement. The gerbilles infected in this manner usually died

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[*] The terms "tsutsugamushi" and "scrub typhus" are considered synonyms for the same disease after the conclusions reached by Lewthwaite and Savoor.[2] The former term is used here because of its priority in the nomenclature of the disease.

[1] Snyder, J. C., Zarafonetis, C. J. D., and Liu, W. T., to be published.

[†] The choice of the name, *R. orientalis*, is based upon the recent re-evaluation of rickettsial terminology by Philip.[3]

[2] Lewthwaite, R., and Savoor, S. R., *Brit. J. Exp. Path.*, 1936, **17**, 448.

[3] Philip, C. B., *Am. J. Hyg.*, 1943, **37**, 301.

[4] Lewthwaite, R., and Savoor, S. R., *Brit. J. Exp. Path.*, 1936, **17**, 1.

in 14 to 19 days, though in one instance death occurred on the 5th day. Of 12 such attempts, 11 were successful in establishing the infection. The one failure occurred in the case of a rabbit from which material taken 2 days previously had successfully infected a gerbille. Following its establishment from infected rabbits into gerbilles, the strain was passed through 15 generations of infection in gerbilles of the species *G. pyramidum*.

The *Imphal 8* branch of this strain was carried through another mouse passage, 3 rabbit passages, and 2 series of 3 gerbille passages (*G. pyramidum*) before storing it frozen at –70°C. One of the 2 series of gerbille passages was maintained by using peritoneal washings for inocula, and the other by using 10% brain suspension.

The *Imphal 13* strain was carried through another mouse passage, as well as 6 passages in *G. gerbillus* and 2 in *G. pyramidum* before being stored frozen at –70°C.

The *Calcutta* strain was maintained for 4 additional passages in rabbits and then established in the species *G. pyramidum* in which it has been carried through 14 successive passages.

The *Ceylon* strain was established directly in *G. pyramidum* from the original infected mouse received in Cairo. Peritoneal washings from the mouse were used to start one series, and a pooled suspension (10%) of its brain and spleen to begin a second series. Three generations of infection were carried out by transferring brain suspensions, and 8 passages have been made with peritoneal washings.

Inocula used and routes of infectivity. As indicated above, 10% suspensions of infectious brain and/or spleen material have been used for passage with success, but, for convenience, peritoneal washings were usually used. About 3-5 cc of peritoneal washings were made from a single gerbille, sacrificed when moribund or freshly dead from the infection. One cubic centimeter of peritoneal washings was inoculated intraperitoneally into each of the next passage gerbilles. This usually killed all the inoculated animals in 9 to 12 days, though deaths occurred as early as 5 days and as late as 36 days after inoculation in isolated instances. Following intraperitoneal inoculation with brain or spleen suspension (10%), the average time of death was delayed 2 to 3 days longer than when peritoneal washings were used.

Most inoculations were made intraperitoneally, though subcutaneous and intravenous routes were tried in a few instances and found to be successful.

Cultures of the various inocula on plain and blood agar plates and in broth media failed to give growth.

The disease in gerbilles. The disease manifestations in these two rodents are essentially the same. Following inoculation, the course is usually not remarkable during the first 8 to 10 days. Towards the latter part of this interval, the infected animal eats less well and there may be ruffing of its fur. Then there appear signs of progressively increasing dyspnea, weakness, and ataxia. Its activity is markedly diminished and it eats poorly or not at all. If the animal is forced to run about in its cage, marked dyspnea and tachypnea result. The difficulty in respiration is perhaps the most prominent feature of the disease. The signs of illness may become gradually accentuated over 2 or 3 days, terminating in the death of the animal, but oftentimes the fatal course is completed in 24 hours or less after the first evidence of illness is noted.

Almost 300 gerbilles have been used in these studies, and all which showed manifestations of illness died. This 100% fatality is due to the virulence of the disease for these rodents as well as to the overwhelming infective dose contained in the inocula used. Titrations of the infectious material have been done twice with the Ceylon strain. In one instance, peritoneal washings in dilutions through 10-3 resulted in the death of all the test animals within 22 days after intraperitoneal inoculation. A second titration using pooled brain and spleen material made up in peritoneal washings was fatal through 10-2, and killed 3 of 4 animals inoculated with 10-3. Thus, the inocula used for the routine passages carried many times the minimal lethal dose.

Autopsy findings in gerbilles. Autopsy almost invariably revealed a sticky peritoneal exudate often up to 1 cc in amount. The liver and especially the spleen were routinely found to be enlarged and covered with a white fibrinous exudate. The other abdominal organs were grossly normal. Collapsed lungs due to a relatively large amount of pleural effusion were generally encountered. There were no other striking findings.

Findings in stained smears of the exudates. Smears of the peritoneal and pleural exudates were usually stained with a simple 1% aqueous methylene blue solution. This was adopted after many attempts with Macchiavello's method had uniformly given the blue stain to the rickettsiæ instead of the basic fuchsin as had been expected from experience with other rickettsial organisms. Giemsa stain was satisfactory, but had the disadvantage of being slower.

Using these staining methods, the peritoneal exudate revealed numerous cellular elements including serosal cells, monocytes, and some lymphocytes and polymorphonuclear leukocytes. The serosal cells frequently exhibited a highly vacuolated cytoplasm. The vacuoles were usually small and numerous, and sometimes indented the margin of the nucleus to give it a punched-out appearance. Within the cytoplasm of these cells could be seen varying numbers of minute pleomorphic rickettsiæ. These appeared at times as minute rods, and at other times as cocci or coccobacilli, occurring singly or in pairs. Less frequently, the organisms were found in the cytoplasm of the monocytes, but the lymphocytes and polymorphonuclear leukocytes were rarely observed to contain them.

The pleural exudate contained similar cell types, but fewer of them. Rickettsiæ were present in some of these cells as well.

It should be noted that the manifestations of all these strains were essentially alike in the experimental animals used. There were no differences in the incubation period, course, average time of death, autopsy findings, or in the appearance of the rickettsiæ in the stained smears of exudates.

Discussion. One of the major difficulties of experimental work with tsutsugamushi disease is the establishment of the identity of the rickettsiæ. The circumstantial evidence pertaining to the 4 strains used in these studies strongly indicates they are *R. orientalis.* All the strains were isolated by experienced workers from patients suffering from an illness clinically diagnosed as tsutsugamushi disease. Their behavior in experimental animals and the microscopic appearance and staining properties of these intracytoplasmic organisms are compatible with the descriptions given for other strains.[4] In addition they fail to grow in ordinary culture media. Cross-immunity tests in gerbilles have not been possible due to the lack of survivors from infection with these strains. The complement-fixation test for tsutsugamushi disease recently described by Bengston[5] should prove to be an important ancillary aid in establishing the relationship of these and other strains, but as yet the antigen is not readily available. For the present, however, it appears to be fairly safe from the evidence cited to conclude that the strains tested in these studies are *R. orientalis.*

Certain potential disadvantages in the use of trapped gerbilles for experimental rickettsial work have been mentioned in a previous communication.[1] These include the occasional occurrence in the blood of both species of extracellular clusters of small rod-like forms which stain red with Macchiavello's method. With this staining method, the latter organisms offer no problem in differentiation from *R. orientalis* which fail to stain with basic fuchsin, but instead hold the methylene-blue counterstain.

Spirillum minus infection, which is occasionally encountered in these rodents, may give rise to confusion, particularly in *B. proteus* OX-K agglutination tests. Savoor and Lewthwaite[5] have described significantly high OX-K agglutination titers in sera from humans and rabbits infected with *Sp. minus.* These workers also cite Felix in stating that wild rodents may often have high OX-19 and OX-K agglutination titers. However, Weil-Felix tests on sera from normal *G. gerbillus* and *G. pyramidum* have failed to give significant OX-19 or OX-K titers in the animals so far tested. The lack of survivors to infection with these strains of *R. orientalis,* however, has prevented the testing of sera from infected gerbilles.

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[5] Bengston, I. A., Pub. H. Rep., U. S. Pub. Health Service, assigned publication date Feb. 11, 1944.

The possibility of naturally occurring infections of these species with *R. orientalis* has been discounted since this disease has never been known to occur in Egypt.

As already indicated, these rodents have been shown to be susceptible to epidemic and murine typhus infection. The finding described herein, namely, that they are also susceptible to tsutsugamushi infection enhances their value as experimental animals. Since infection results in death of these rodents, they are particularly suitable for titrations of tsutsugamushi inocula, thus making possible their use in vaccine assay, in neutralization tests, and in the trial of therapeutic drugs. They are satisfactory animals for strain maintenance and yield a rich source of infectious material. Their value in strain isolation cannot be estimated as no attempts have yet been made with them. They can, of course, receive a greater amount of inoculum than may be placed in the anterior chamber of a rabbit's eye. Furthermore, it was found that a much higher percentage of successful transfers resulted from rabbit-to-gerbille than from rabbit-to-rabbit passages. This made the gerbilles much more useful, both as for certainty of infection and as a richer source of infectious material for further passage.

It is not intended to imply, however, that these animals possess any great superiority over laboratory-bred white mice for the study of tsutsugamushi disease. Rather, *G. pyramidum* and *G. gerbillus* were discovered to be suitable substitute animals in the absence of a supply of white mice.

Summary. Experimental evidence indicates that the two rodents, *Gerbillus gerbillus* and *Gerbillus pyramidum* are readily susceptible to large doses of scrub typhus rickettsiæ. One hundred percent of the animals succumbed to the disease when inoculated by the intraperitoneal, subcutaneous, or intravenous route. Smears of the peritoneal and pleural exudates showed varying numbers of minute phomorphic rickettsiæ within the cytoplasm of the cells. It was found that a much higher percentage of successful transfers resulted from rabbit-to-gerbille than from rabbit-to-rabbit passages. For this reason gerbilles were found to be much more useful both as for certainty of infection and as a richer source of infectious material for further passage than rabbits. They are suitable substitute animals in the absence of a supply of white mice.

The author wishes to express his appreciation to Lt. Col. M. H. P. Sayers, R.A.M.C., for making available to them the Imphal and Calcutta strains; Major M. T. Parker, R.A.M.C., for the Imphal 8 and Imphal 13 strains; and Dr. S. R. Savoor for the Ceylon strain.

The assistance of Sgt. J. Dworkowitz and Corporals D. Hogan, R. Stearman, and R. Cassell in these studies is gratefully acknowledged.

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Evidence that Menstrual "Toxin" and Canine "Necrosin" are Identical

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The similarities between the toxic material in menstrual discharge[1] and the more recently described injury factor, termed "necrosin," of pleural exudates[2] suggested to us that these substances might be identical.[3] It is merely for the sake of brevity that either is referred to as a single substance, since neither has been purified to any degree, but the further investigations herein reported establish many points of similarity and give evidence that both contain an identical toxic protein.

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[1] Smith, O. W., and Smith, G. V., PROC. SOC. EXP. BIOL. AND MED., 1940, **44**, 100.

[2] Menkin, V., *Science*, 1943, **97**, 165.

[3] Smith, O. W., and Smith, G. V., PROC. SOC. EXP. BIOL. AND MED., 1944, **55**, 285.

Materials. Menstrual discharge has been obtained from normal women by means of rubber cups. Emphasis has been placed on refrigerating and using it as soon as possible after collection.

Pleural exudate from dogs following the injection of turpentine has been our source of necrosin.[4] Dr. Menkin kindly supplied us with a limited amount for our earlier experiments, the rest having been prepared by us. The fluid from the first tap, 24 hours after turpentine, usually clots soon after removal. At the 48-hour tapping it may or may not clot. When clotting occurs the pH is around 7 or over. After 48 hours the exudate does not clot. The pH of exudates that fail to clot is under 7, usually 6.4 to 6.8.

Similarities between menstrual "toxin" and canine necrosin. 1. Death of 19- to 21-day-old male or female rats within 48 hours after subcutaneous injection has been our criterion of toxicity. The toxicity of menstrual discharge is most concentrated in the endometrial debris which separates as a middle layer, upon centrifugation, between the red blood cells and the "serum." It is proportional to the amount of debris which is greatest when the rate of flow is slow and the pH of the fluid around 6.8.

The toxicity of whole canine pleural exudate differs greatly with different specimens and is rarely as marked as in menstrual discharge. The most toxic exudates are usually obtained 48 to 96 hours after the injection of turpentine when the fluid is thin and cloudy, does not clot, and has a pH around 6.8 or under. After centrifugation, the cellular debris which settles is about 10 times as toxic as the supernatant fluid. Toxicity is proportional to the amount of this debris. In both exudate and discharge, then, the toxicity is most concentrated in, and presumably derived from, tissue elements.

2. If, after centrifugation, the clear "serum" of menstrual discharge and the supernatant exudate are treated by methods which precipitate the euglobulins, the toxicity is concentrated in this fraction, indicating that both toxin and necrosin either are proteins of large molecular size or associated with such proteins. They are, however, atypical, compared with the euglobulin fraction of normal venous serum, being insoluble in physiological saline solution. With some specimens the water-insoluble portion after dialysis of the euglobulin precipitate from ammonium sulphate treatment forms a heavy suspension in physiological saline solution which is not thrown down when centrifuged at 3,500 r.p.m. We have noted this more often in preparing necrosin than menstrual toxin. When the supernatant fluid is clear, it is practically non-toxic.* Throughout this communication the terms menstrual "toxin" and "necrosin" refer to a suspension in physiological saline of the water-insoluble portion after dialysis of the euglobulin precipitate.

3. The toxicity of both discharge and exudate is greatly diminished or destroyed by heat, exposure to air, and changes in pH. Partially purified preparations, even in the dry state, are particularly labile. They may lose toxicity in the process of drying, even during dry-freezing. Even if ammonium sulphate is added to fresh materials to one-third saturation and they are refrigerated in this state until use, the toxicity is much diminished and sometimes entirely lost after 2 to 4 weeks. This lability has hampered attempts at purification, standardization, and immunization.

4. The pathological findings in a rat that has received a lethal dose of necrosin are

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[4] Menkin, V., *Arch. Path.*, 1943, **36**, 269.

* Menkin has recently reported[5] further purification of necrosin by removing from the ammonium sulphate euglobulin precipitate that portion which is insoluble in water in the presence of sulphate ions. In our experience there is no insoluble portion at this stage except when whole exudate and discharge are processed, in which case the material which does not dissolve if water is added consists entirely of debris. Menkin has stated that this residue, after repeated washings with distilled water, is pyrogenic but non-toxic. Our only explanation for this is that the washing of the debris with ordinary distilled water, which is acid, greatly reduces its toxicity.

[5] Menkin, V., *Arch. Path.*, 1945, **39**, 28.

entirely similar to those described following the injection of menstrual toxin.[1] Furthermore, Dr. Menkin, while in this laboratory, found that the intracutaneous injection of our toxin in the rabbit elicited the same local response as necrosin.[2]

5. Menstrual discharge and necrosin[2] both hasten the clotting of blood.

6. We have amply confirmed Menkin's observation[6] that canine necrosin, given intravenously, is pyrogenic in the rabbit, very small quantities causing a 2.5 to 5°F rise in rectal temperature about 3 hours after injection. Menstrual toxin gives the same type of curve, even in the minute amounts that must be used to avoid killing the animals. The pyrogenic factor of both exudate and discharge is concentrated in the euglobulin fraction. The induction of fever is apparently not due to a foreign or non-specific protein reaction or to the intravenous injection of a suspension. It is equally marked when filtered menstrual serum or exudate is given and has failed to occur with saline suspensions of dried human placenta or preparations of exudate which had lost toxicity in the process of drying.

7. Menkin has referred to unpublished data indicating the presence of proteolytic activity in the necrosin fraction of canine pleural exudate.[5] Using a simple technic,[7] we have consistently been able to demonstrate complete dissolution of a fibrin clot with menstrual toxin[8] and have found 3 different active preparations of canine necrosin to be as markedly fibrinolytic as the human material. The enzyme is not demonstrable in the euglobulin fraction of normal human serum. Specimens that have lost toxicity may completely retain fibrinolytic activity, indicating that the toxin and enzyme are not the same.

Identity of menstrual toxin and canine necrosin. 1. It has been shown that repeated injections of menstrual toxin[3] or necrosin[2] into rabbits induce precipitin antibodies. Since, of course, neither material is a pure protein, the antibodies may have been non-specific. If, however, the serum of a rabbit immunized against menstrual toxin precipitated canine necrosin and *vice versa,* the evidence for an identical factor in these materials of different origin in different species would be quite definite. We have found this to be the case.

The sera of 3 rabbits with precipitin titers against menstrual toxin of 1:1000 or over consistently gave equally high titers against 8 samples of active canine necrosin but negative tests against 8 other preparations of canine necrosin in which no toxicity was demonstrable. Moreover, the sera of 10 rabbits immunized against canine necrosin to a titer of 1:1000 or over gave equally strong reactions with 12 specimens of active menstrual toxin but negative tests with 3 non-toxic samples.

For further controls, 9 sera from normal women and 2 from a normal male were tested against 3 of the canine necrosin-immune rabbits. All were negative. Control tests with normal canine serum against that of menstrual toxin-immune rabbits introduced an enigma. With 5 of 11 sera from 8 apparently healthy dogs, positive precipitin reactions were obtained. These same canine sera gave negative tests against normal rabbit serum, normal human serum, and that of a rabbit with a titer of 1:100,000 against normal human serum, thus appearing to rule out natural antibodies as an explanation. Obtaining unexpected positive tests with normal canine serum makes us wonder whether or not dogs may not often have the injury factor in their circulation, *e.g.,* from heartworms,[9] which are a common affliction of experimental dogs in this vicinity.

2. We have shown that the serum of rabbits immunized with menstrual discharge prolongs the survival of immature rats given a lethal dose of menstrual discharge.[3] This serum has also been found to delay significantly death from canine necrosin. The protective factor is concentrated in the pseudoglobulin fraction. To obtain even this partial protection required a precipitin titer of at least 1:1,000,000 in the immunized rabbits. We have not yet been able to induce so high a titer in rabbits immu-

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[6] Menkin, V., *Federation Proc.*, 1944, **3**, 32.

[7] Tagnon, H. J., Davidson, C. S., and Taylor, F. H. L., *J. Clin. Invest.*, 1942, **21**, 525.

[8] Smith, O. W., and Smith, G. V., *Science,* in press.

[9] Phillips, J. H., *Am. J. Hygiene*, 1939, **29**, 121.

nized against canine necrosin. Presumably because of this, we have gotten transferable protection against menstrual toxin with only 4 of 12 specimens of necrosin-immune rabbit serum.

The evidence presented in favor of the identity of menstrual toxin and Menkin's necrosin supports the hypothesis that the production of a specific toxic protein may be a universal result of cellular injury. On the basis of recent work,[8,10] we now have the concept that tissue damage releases a proteolytic enzyme and that enzymatic action yields the toxic protein.

Conclusion. Physical, chemical, and biological similarities, cross precipitin tests and experiments on transferable immunity indicate that Menkin's so-called necrosin of canine pleural exudate and the toxin of human menstrual discharge are identical.

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[10] Mirsky, A., and Freis, E. D., PROC. SOC. EXP. BIOL. AND MED., 1944, **57**, 278.

15000

Evidence for Circulating Menstrual "Toxin" During Menstruation and Toxemia of Late Pregnancy.

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We have suggested that menstrual "toxin," a by-product of the change in the endometrium which follows withdrawal of hormonal support, may be responsible for the local vascular phenomena which characterize menstruation and that the same or a similar toxin from the uterus may cause the similar but generalized vascular damage in toxemia of late pregnancy, this disease also being associated with hormone withdrawal.[1] We have been unable to obtain from the circulating blood of menstruating or toxemic women a factor lethal to immature rats. The findings presented in the preceding paper,[2] however, have provided more sensitive tests for this material. Its pyrogenic and fibrinolytic activity may be demonstrated with minute amounts. Furthermore, the fact that canine pleural exudate contains an identical substance supplies a highly specific test in the form of cross precipitin reactions with canine "necrosin"-immune rabbit serum.

Both menstrual "toxin" and "necrosin," injected intravenously into the rabbit, cause a typical rise in rectal temperature, a maximum elevation of 2.5 to 5°F being observed 3 to 4 hours after injection.[2] In testing for pyrogenicity, readings are made just before and at hourly intervals for 7 hours after injection. In order to avoid any possibility of anaphylaxis, we plan to use an animal repeatedly at no more than 4-day intervals for a series of tests and then let it rest for at least 8 weeks. Half of a cc of unextracted serum from the venous blood of women was injected. Sixteen sera from normal nonmenstruating women were tested. The average rise in temperature was 0.9°F. In 10 of these there was no more than a 1° rise at any time. In the other 6 a 1.6° to 2.6° elevation occurred 1 to 2 hours after injection, but, unlike the curve after menstrual "toxin" or "necrosin," was declining by the time of the 3-hour reading. Thirteen tests were run using serum from patients normally pregnant between the thirty-fourth week and term. The average rise in temperature was 1.1°F. In all but 2, the tests were negative; that is, there was either no fever at all or the readings at 3 hours and thereafter were lower than those at 1 to 2 hours following injection. The sera from 2 of the normally pregnant patients gave the response typical of the toxin; that is, an elevation of 2.5 to 3.4°F reaching a peak at the 3- to 4-hour reading. With 13 specimens taken during menstruation the average rise in temperature was 2.5°F. Nine elicited a maximum elevation of 1.7 to 4.8°F at 3 to 5 hours after injection. The other 4 failed to give the response typical of menstrual "toxin" and "necrosin." With 10 specimens from patients with pre-eclampsia or eclampsia, the average rise was 2.7°F. Seven gave a 2.4 to 4.5°F rise with the typical 3- to 4-hour peak and 3 gave no significant change in temperature. Sixteen of 23, or 70%, of the tests with sera of toxemic or menstruating women, therefore, were positive, whereas only 2 of 29, or 7%, of the control sera resulted in a similar response.

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[1] Smith, G. V., and Smith, O. W., *J. Clin. Endocrinol.*, 1941, **1**, 470.

[2] Smith, O. W., and Smith, G. V., PROC. SOC. EXP. BIOL. AND MED., 1945, **59**, 116.

Menstrual "toxin" and "necrosin" are markedly fibrinolytic.[2] Evidence for fibrinolytic activity in the circulating blood at the time of menstruation, in late pregnancy toxemia, and in post-operative morbidity has been reported.[3] In these preliminary experiments, unextracted serum was employed and oxalated plasma used as the source of fibrinogen. We have since changed our technic, using a purified preparation of fibrinogen* as well as thrombin* and prolonging the period of observation to 24 hours at 37°C. We have also concentrated the sera by dialyzing 5.0 cc against running tap water for 5 hours, and taking up the euglobulin precipitate in 0.8 cc of saline for test. To this, 0.1 cc of an approximately 1% solution of fibrinogen and 0.1 cc (1 U) of thrombin have been added. With this technic, 6 sera from non-menstruating women and 6 from normal pregnancies have been tested. No measurable dissolution of the clot occurred in any, although there was slight retraction from the sides of the tubes by the end of the 24-hour period. Of 9 sera from the venous blood of menstruating women, 2 showed complete dissolution and the other 7 contained only a slight residual clot, 0.7 to 0.9 cc of fluid being present in each tube at the end of 24 hours. The results of 7 similar tests on the venous sera of 6 patients with late pregnancy toxemia and 1 with eclampsia were even more striking, complete dissolution of the clot being observed in every instance.

The identity of menstrual "toxin" and canine "necrosin" was established by the cross precipitin experiments with immune rabbit serum reported in the preceding paper.[2] If this same factor is present in the circulating blood of menstruating and toxemic women, their serum should show positive precipitin reactions with canine "necrosin"-immune rabbit serum. We have tested 9 sera from normal non-menstruating women (5 were pregnant), 4 from menstruating women, and 4 from patients with toxemia of late pregnancy against the sera of 3 rabbits injected repeatedly with canine "necrosin" to a precipitin titer of 1:1000 or over. Negative results were acquired with all 9 of the control sera, whereas the other 8 all gave titers of 1:1000 or over. Four of these positive sera (3 from menstruating women and 1 from a patient with pre-eclampsia) gave negative tests after standing 2 to 3 weeks in the refrigerator. This is in accord with the known lability of the toxic factor and further substantiates the specificity of this test. It would appear that a substance identical to menstrual "toxin" and canine "necrosin" is present in the circulating blood of menstruating and toxemic women.

One of our original purposes in looking for a substance similar to menstrual "toxin" in other than human material was to obtain, for possible clinical use, an immune serum from which antibodies to non-specific human proteins would be eliminated. The difficulties encountered in our attempts to protect rats against menstrual "toxin" with "necrosin"-immune rabbit serum have been reported in the preceding paper.[2] Of even greater promise, however, from the therapeutic angle has been the finding that a protective substance is always present in the same material that contains the toxic factor and appears to be concentrated in the pseudoglobulin fraction. These fractions were prepared by half saturation of the material with $(NH_4)_2SO_4$ after removal of the euglobulins either by dialysis or by one-third saturation with $(NH_4)_2SO_4$. In the experiments reported, the equivalent of 2.5 to 5.0 cc of the original material was used for each test. Following the previously published technic,[4] we have been able to demonstrate consistently a significant prolongation of survival time of rats after the injection of a lethal dose of toxin by the simultaneous administration of the pseudoglobulin fractions of menstrual discharge (6 experiments), of canine pleural exudate (3 experiments), and of human pleural exudate from metastatic cancer of the breast (3 experiments, in all of which protection against death was complete). Furthermore, in 19 experiments with the pseudoglobulin fractions of venous sera collected during menstruation and in 7 with the pseudoglobulins of sera from patients with toxemia of late pregnancy, definite protective action was demonstrable, death being completely prevented in 5 instances when pseudoglobulins of venous sera from menstruating women were given. In 14 control tests, no protection against a lethal dose of menstrual toxin was afforded by the administration of pseudoglobulin fractions of sera from normal males, normal non-menstruating females or normally pregnant women or of the ascitic fluid from a patient with hepatic cirrhosis. Concentrated immune serum globulins from normal human blood* were also ineffective. The therapeutic possibilities of these findings in toxemia of pregnancy are apparent. Since the "toxin" appears to be a product of cellular injury from tissue damage other than that involving hormone withdrawal,[2] even more generalized clinical implications are involved. The nature of the protective substance remains obscure, but the fact that it is apparently found only in association with the "toxin" supplies further evidence in favor of the presence of this "toxin" in the circulating blood of menstruating and toxemic women.

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[3] Smith, O. W., and Smith, G. V., *Science*, in press.

* The fibrinogen, thrombin, and immune serum globulins employed in these investigations were kindly supplied by Prof. E. J. Cohn and Dr. J. T. Edsall of the Department of Physical Chemistry, Harvard Medical School, Boston, Massachusetts. These products were prepared from blood collected by the American Red Cross, under a contract recommended by the Committee on Medical Research, between the Office of Scientific Research and Development and Harvard University.

Conclusion. Serum from the circulating blood of women during menstruation and toxemia of late pregnancy differs from that of normal non-menstruating and normally pregnant women and resembles menstrual "toxin" in that it is pyrogenic and fibrinolytic, gives positive precipitin tests with the serum of rabbits immunized against canine "necrosin," and contains a pseudoglobulin capable of prolonging the survival time of rats after a lethal dose of menstrual toxin. It is concluded that a factor identical to menstrual "toxin" is present in the circulating blood of menstruating and toxemic women. The protective factor is being further investigated as to its possible therapeutic applications.

We are indebted to Dr. William J. Mulligan of the Boston Lying-in Hospital for obtaining blood from pregnant patients and to the nurses of the Free Hospital for Women for donating their blood for the studies on non-pregnant women.

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[4] Smith, O. W., and Smith, G. V., PROC. SOC. EXP. BIOL. AND MED., 1944, **55**, 285.

15001

Growth of Mammary Glands of Hypophysectomized Rats Following Estrogen and Lactogen Administration.*

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It is well established that estrogens will stimulate duct growth of mammary glands in either normal or castrated animals (Turner[1]). Estrogens, however, are unable to cause growth of the mammary duct system in rats following ablation of the hypophysis and therefore a pituitary factor appears to be involved. In addition there is evidence that the pituitary factor is of a protein nature and that it may be the lactogenic hormone. The literature on this subject has been reviewed by Mixner and Turner.[2]

We have been interested in determining the effectiveness of estrogen in combination with the lactogenic hormone in producing mammary duct growth in the hypopyhysectomized rat. Previous experiments in the rat[3] were negative, but positive results were obtained in the mouse[4] and in the guinea pig.[5]

Forty-eight castrated and hypophysectomized rats were used in the study here reported. In general both operations were performed at the same time but in some instances a short interval occurred between operations. Body weight at the time of hypophysectomy ranged between 72 and 167 g.

In all cases, 5 days was allowed to elapse after hypophysectomy. The hormone or hormones were injected subcutaneously once daily for 10 days (in most cases), and the animals were autopsied 24 hours after the last injection. All pituitary capsules were serially sectioned, stained with Mallory's connective tissue stain, and examined microscopically. Only data from completely hypophysectomized rats are presented. At autopsy the right abdominal mammary gland was removed from each rat. These glands were stained and mounted *in toto*. The mammary glands were examined under a dissecting microscope and rated on the basis of response. Glands with either thin and bare ducts or thin ducts and a few regressing lateral buds were given a negative rating (—); a one plus (+) rating was assigned glands with lateral and end buds and glands with a thickened duct system either with or without lateral buds; and a two plus (++) was given to glands showing an extension of the duct system with enlarged end buds.

Estradiol dipropionate (Progynon—DP†) was the estrogen of choice; however, 3 different lactogenic hormone preparations were used. These preparations are designated as follows: lactogen A—a purified hormone (Prolactin—Schering†) containing 35 I.U. per mg; lactogen B—a purified hormone (Schering†) containing 14 I.U. per mg; and lactogen C—a purified hormone (Difco Prolactin‡) containing 25 I.U. per mg.

The experiments are considered by groups.

Group I. Three rats received no injections. Their mammary glands consisted of a thin and bare duct system (—).

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* Journal Series Paper of the New Jersey Agricultural Experiment Station, Rutgers University, Department of Dairy Husbandry.

[1] Turner, C. W., *Sex and Internal Secretions*, 1939, Chapter 11 (E. Allen, ed.), Williams and Wilkins Co., Baltimore.

[2] Mixner, J. P., and Turner, C. W., *Mo. Agr. Exp. Sta. Res. Bull.*, 1943, 378.

[3] Nelson, W. O., and Tobin, C. E., *Anat. Rec.*, 1936, **67** (Suppl. 1), 111.

[4] Gardner, W. U., and White, A., Proc. Soc. Exp. Biol. and Med., 1941, **48**, 590.

[5] Gomez, E. T., *Endocrinology*, 1942, **31**, 613.

† The estradiol dipropionate (Progynon-DP) and two lactogenic hormone preparations (Prolactin-Schering and Schering) were generously supplied by Dr. E. Schwenk of the Schering Corp., Bloomfield, N.J.

‡ One lactogenic hormone preparation (Difco Prolactin) was generously supplied by Dr. Robert W. Bates of Difco Laboratories, Inc., Detroit, Mich.

TABLE I.
Mammary Gland Growth in Hypophysectomized Rats Following Estrogen and Lactogen Administration.

Group	Treatment		Test period (days)	No. of rats	Mammary† response		
	Estrogen, μg	Lactogen, I.U.			—	+	++
I	Control		10	3	3		
II	1*	0	10	9	9		
III	0	35-A‡	9-10	4	2	2	
IV	1*	35-A	8-10	8	3	4	1
V	1		7-10	7	7		
VI	0	70-B§	10	2		1	1
VII	0	70-C¶	10	2		2	
VIII	1	70-A	7-10	3		2	1
IX	1	70-B	10	2			2
X	1	70-C	10	8			8

* Injected every other day.

† — = glands with either thin and bare ducts or thin ducts and a few regressing lateral buds; + = glands with lateral and end buds and glands with a thickened duct system either with or without lateral buds; and ++ = glands showing an extension of the duct system with enlarged end buds.

‡ A—A purified hormone (Prolactin-Schering) containing 35 I.U. per mg.

§ B—A purified hormone (Schering) containing 14 I.U. per mg.

¶ C—A purified hormone (Difco Prolactin) containing 25 I.U. per mg.

Group II. Nine rats were injected with 1 μg of estrogen every other day for 10 days. None of the mammary glands showed signs of growth (—), all glands showing involutionary changes. Six of the 9 glands were similar to those of the controls, whereas in the remaining 3 glands a few lateral buds were still present.

Group III. Four rats received daily for 9-10 days 35 I.U. of lactogen A. Two of the mammary glands were similar to those of the controls (—), whereas the remaining 2 glands had lateral buds and a slightly thickened duct system (+).

Group IV. Eight rats received 35 I.U. of lactogen A daily for 8-10 days and 1 μg of estrogen every other day. The results are inconsistent. Three glands had not been stimulated (—), 3 showed a thickened duct system with many lateral buds (+), one showed many lateral and end buds (+), and one gland exhibited growth similar to that seen in the intact rat following estrogen treatment (++).

Group V. Seven rats were injected with 1 μg of estrogen daily for 7-10 days. The mammary glands of 5 rats consisted of a bare duct system (—); and, in the remaining 2 rats the glands were involuting, though lateral buds were still present (—).

Group VI. Two rats received 70 I.U. of lactogen B daily for 10 days. The glands from both rats showed a slightly thickened duct system, one gland having many lateral buds (+) and the other gland numerous lateral buds as well as proliferating end buds (++).

Group VII. The daily injection of 70 I.U. of lactogen C for 10 days into 2 rats caused a slight thickening of the ducts which were without buds (+).

Group VIII. Three rats received 70 I.U. of lactogen A and 1 μg of estrogen daily for 7-10 days. Lateral buds were present in 2 of the glands (+), and the third gland showed numerous lateral buds and enlarged end buds (++).

Group IX. The mammary glands of 2 rats injected daily for 10 days with 70 I.U. of lactogen B and 1 μg of estrogen showed stimulation similar to that seen in intact rats following estrogen injections (++).

Group X. The daily injection of 70 I.U. of lactogen C and 1 μg of estrogen into 8 rats for 10 days produced duct growth of the mammary glands in each instance. Five of the mammary glands exhibited enlarged end buds and numerous lateral buds (++). Enlarged end buds were observed in the remaining 3 glands (++), otherwise the ducts were bare. Two of these 3 glands were from rats that had lost weight during the experiment. This latter condition of the mammary glands is similar to that observed by Reece and Leonard[6] following either estrogen injection into partially hypophysectomized (minute fragment) rats or estrogen and growth hormone injections into hypophysectomized rats. The results are summarized in Table I.

These results indicate again that estrogens will not stimulate duct growth of the mammary glands of hypophysectomized rats. The main action of lactogen, when injected alone, appears to be that of causing a thickening of the duct system along with a tendency to prevent the regression of the lateral buds. In a single case, lactogen injection caused end buds to proliferate. Our observations suggest that the lactogenic hormone is the pituitary factor which permits mammary duct growth in the rat. These observations agree with other known facts, since it has been shown that the lactogen content of the pituitary gland increases considerably at sexual maturity,[7] a time when the mammary duct system is rapidly proliferating. Moreover, estrogen injections into ovariectomized rats augment the lactogen content of the pituitary gland,[8] making possible the development of the duct system.

Summary. In castrated-hypophysectomized rats estrogen did not stimulate mammary duct growth. The injection of lactogenic hormone caused a thickening of the duct system and in combination with estrogen it induced mammary duct growth similar to that seen in intact rats following estrogen injections.

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[6] Reece, R. P., and Leonard, S. L., *Endocrinology*, 1941, **29**, 297.

[7] Reece, R. P., and Turner, C. W., *Mo. Agr. Exp. Sta. Res. Bull.*, 1937, 266.

[8] Reece, R. P., and Turner, C. W., PROC. SOC. EXP. BIOL. AND MED., 1937, **36**, 283.

15002

Serologic Agglutination of Antigen-Coated Dye Particles.

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Previous reports[1,2] have indicated the possible utility of a serologic reaction wherein the antigen as *e.g.*, horse serum or neurotropic virus, has been adsorbed to heat killed bacterial cells (*Serratia marcescens*) and in this form, employed as an agglutinable suspension. The technic of the test (B.A. or bacterial agglutination method) in its present state of development[3] is one, which for satisfactory operation, particularly with viral antigens, requires that scrupulous attention be given to various details in its performance. Some difficulty may be experienced also in observing a reaction, even under optimal conditions, since the masses of agglutinated bacteria are very small and may not be easily recognized. It was considered that certain of the critical limitations or deficiencies of the B.A. method could be obviated by substituting for *S. marcescens* cells, other types of particulate matter possessing similar adsorptive capacities but of units larger in dimension. Also it was expected that a particulate substance might be found which would serve the purpose without involving some of the disadvantages encountered in the use of collodion pellets[1] as for instance, the necessity for careful removal of electrolyte from antigenic material, by dialysis, as a preliminary to its adsorption. Preliminary experiments indicated that particles of carmine[4] possessed this advantage when used with horse serum as the antigen and in the experiments covered in the present report suspensions of this dye have been used as the particulate material.

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[1] Roberts, E. C., and Jones, L. R., PROC. SOC. EXP. BIOL. AND MED., 1941, **47**, 11.

[2] Roberts, E. C., and Jones, L. R., PROC. SOC. EXP. BIOL. AND MED., 1942, **49**, 52.

[3] Roberts, E. C., *J. Immunol.*, 1945, **50**, 55.

In coating the particles of carmine with horse serum the routine technic has been developed after a great many preliminary experiments dealing with various factors which are involved in satisfactory performance of this type of test. Among these factors are that of particle size, ratio of particles to added antigen, dilution, time, temperature, pH and electrolyte concentration.[3] The technic, as it was finally developed, may be described as follows: 2 g of the dye suspended in 10 ml of water are ground in a mortar for 4 hours, whereupon, the suspension is centrifuged for one minute in a 52° angle centrifuge at 1750 R.P.M. (R.C.F. approximately 350). The sediment, which consists of the coarser particles, is discarded and the supernate is re-centrifuged for 6 minutes at 2750 R.P.M. (R.C.F. approximately 900). The sediment which is now collected contains particles of dye of about the proper size for satisfactory behavior in subsequent use. (Larger particles of the dye have been found to be less suitable because of greater sensitivity to the agglomerating effect of electrolyte.) A 1% suspension (by volume) is made in 0.25% sodium chloride solution which constitutes the stock suspension and has been found to keep well even upon prolonged storage. To "coat" these particles with the antigen, 4 ml of a 1:20 dilution of normal horse serum in 0.85% salt solution are added to 1 ml of the suspension; the mixture is gently shaken for 15 minutes at room temperature whereupon it is centrifuged at a rapid rate of speed, to insure complete sedimentation of the sensitized particles. The supernate is discarded and the sediment resuspended evenly in 5 ml of 0.25% salt solution. The antigen suspension is now ready for use and when stored at 5°C retains its usefulness for long periods of time. Each lot of suspension may be tested for specific agglutinability with serial dilutions of a standard antihorse serum. In our experience with various lots prepared at different times, the uniformity, as determined in this way, has been striking.

In making agglutination tests with antigen suspensions as prepared above and rabbit antihorse serum, we have found it convenient and satisfactory to employ thick glass slide with multiple depressions.* One drop of antigen suspension is placed in each depression and one drop of the antihorse serum (or a desired dilution thereof in 0.85% salt solution) is added, whereupon the slide is placed on the stage of a mechanical shaker, operating at a speed of 145 oscillations per minute, for 15 minutes and at room temperature. Suitable controls are included in each series of tests. Agglutination of the sensitized particles under these conditions is sharp, well defined and easily recognized. While with particles of this size the tests may be made in tubes, longer incubation periods in the water bath with higher temperatures are required and the end-titer of an antiserum is not increased. In the test tube method several hours of incubation at 28°C, or 43°C, are required for maximal agglutination which on the other hand occurs in 15 minutes when the open slide method is used in conjunction with the mechanical shaker.

When the sera of rabbits, previously injected with a single immunizing dose of horse serum are examined for the first appearance of specific antibody, employing agglutination of antigen-coated-carmine-particles (AACCP) wherein serial dilutions of the antiserum are used, and for comparison the conventional precipitation test employing serial dilutions of antigen, it has been found, as is indicated in Table I, that antibody can be detected from 2 to 6 days earlier by the former method. This early detection of antibody appears to be a particular merit of the AACCP method. However, as antibody production advances in a given animal the titer of the antiserum as expressed in terms of antigen dilution, is usually higher by the conventional precipitation method.

The validity of the AACCP method for

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[4] Smorodintzeff, A. A., and Fradkina, R. V., Proc. Soc. Exp. Biol. and Med., 1944, **56**, 93.

* The type commonly used in making the Kline test for the diagnosis of syphilis.

TABLE I.
Antibody Response in Rabbits Injected with One Dose of Horse Serum.

Rabbit No.	Method	Pre-injection	Post-injection				
			2 da.	4 da.	6 da.	8 da.	12 da.
(0.5 ml/kg intravenously)							
33	AACCP*	—	—	20	20	20	40
	Pp†	—	—	—	2	40	10240
34	AACCP	—	20	80	80	640	80
	Pp	—	—	—	2	5120	20480
43	AACCP	—	—	2	80	40	
	Pp	—	—	—	5120	20480	
44	AACCP	—	40	80	80	320	
	Pp	—	—	—	—	1280	
45	AACCP	—	—	2	2	40	
	Pp	—	—	—	—	1280	
46	AACCP	—	20	2	20	40	
	Pp	—	—	—	—	2560	
(10 ml/kg subscapularly)							
35	AACCP	—	20	40	80	160	1280
	Pp	—	—	320	1280	10240	20480
36	AACCP	—	20	40	80	160	1280
	Pp	—	—	320	640	5120	10240

* Agglutination of antigen (horse serum) coated carmine particles. Figures refer to the highest dilution of antiserum at which reaction occurred.

† Conventional precipitation reaction. Figures refer to highest dilution of antigen yielding a visible precipitate.

TABLE II.
Effect of Horse Serum (Antigen) on Carmine Particles Sensitized with Various Rabbit Antihorse Sera.

Antiserum	AACCP titer of antiserum	Dilutions of horse serum (antigen)						
		1:2	1:5	1:10	1:20	1:40	1:80	1:160
No. 1	1:5120	xx	xx	xx	xx	x	x	—
No. 2	1:1280	xx	xx	xx	x	x	—	—
No. 3	1:20	—	—	—	—	—	—	—

the detection of humoral antibody would seem to be established since the agglutinating factor is not encountered in the pre-immunization sera of rabbits nor in the sera of rabbits immunized against non-related antigens. Further, in adsorption experiments wherein antigen-coated particles were added to homologous antisera it was found that all of the agglutinating factor (antibody) could be specifically removed.

In a limited number of experiments devoted to a study of the behavior of dye particles when used for this purpose it was found that antigen-sensitized particles are much less sensitive to the agglutinating effect of sodium chloride than are non-sensitized ones and accordingly the threshold concentration of electrolyte required for agglutination of the former appears to be related, quantitatively or otherwise, to the extent or character of the adsorption of horse serum antigen. If a particular suspension of antigen-coated particles agglutinated upon the addition of an equal volume of 3.4% sodium chloride (*i.e.*, 4 × 0.85) it was arbitrarily considered to be unsatisfactorily coated, to be poised too close to the electrolyte threshold at which non-specific agglutination would occur, and accordingly was not employed in making tests. Other preliminary experiments revealed that carmine dye particles are susceptible to sensitization with both the albumin and globulin fractions of horse serum since particles sensitized with either of these fractions were agglutinated by antihorse serum. Also, it was observed that the process could be reversed, *i.e.*, particles could be satisfactorily coated with rabbit antihorse serum (antibody) and later agglutinated by the mere addition of horse serum (antigen.) The results of typical titrations are given in Table II. It will be noted also that the ability of a given antiserum to sensitize carmine particles under these conditions is related to the antibody content of the serum employed.

With respect to the adsorption of antibody, the behavior of carmine particles differs possibly from that of collodion particles since Jones[5] has reported experiments wherein the latter were exposed to an immune serum, subsequently washed with alkaline salt solution, and later failed to agglutinate upon the addition of antigen. The difference in the ability of collodion particles to become sensitized with antibody as compared with that of carmine particles may be due to differences in the composition of these substances. On the other hand, in the experiments with collodion particles, antisera of less than adequate potency may have been used, or the triple washing with alkaline salt solution may have resulted in the elution of a significant amount of antibody.

In our experiments all rabbit blood specimens were allowed to clot, placed in the refrigerator without delay and the sera were removed and tested without heat inactivation. The tests were made usually on the same day, but in no instance more than 40 hours later. Accordingly, all specimens might be regarded as having been fresh ones. During the study of the AACCP reaction it became evident that there are certain differences in the behavior of fresh and heat inactivated sera. Experiments with this aspect of the study may be summarized as follows: the earliest antibody factor which appears after administration of antigen (often occurring in the 48 or 96-hour post-injection sample) and which is detectable only by the AACCP method, appears to be relatively heat labile (withstands 52°C, 30 minutes; destroyed by 56°C, 30 minutes) and to be susceptible to deterioration upon standing (4 days at 5°C). Later increments of the antibody response in a given animal, as observed in subsequent serum samples, are characterized by usual degree of stability to heat and aging, however, a labile antibody-factor is also present in these specimens. In our experiments it has not been possible to re-activate such heated or stored sera which originally possessed the labile antibody-factor by the inclusion of fresh normal serum in AACCP tests. On the other hand, as might be expected, the antibody-factor (precipitin) involved in the conventional precipitation reaction, appearing somewhat later in these immunized rabbits, was not affected by heat or storage. Further study is required to elucidate the full significance of the above observations.

The sensitivity of the AACCP method is probably attested to by the observation that it will detect antibody earlier in the course of the immunization procedure than will the conventional precipitation test employing the antigen dilution method. Further, the former method will permit the detection of antibody in a relatively high dilution of antiserum as compared to the inability of such antisera to cause visible precipitation when diluted beyond reasonably low levels. For example, precipitating antisera which fail to elicit a visible reaction in the precipitation test when diluted above 1:10 or 1:20 may manifest the AACCP reaction when diluted 1:1000 or higher. This particular aspect of the AACCP reaction is in accordance with the concept of the mechanics of flocculation as afforded by Zinsser[6] wherein it is noted that larger particles present much less surface area per unit of mass than smaller ones. Since antigen-antibody reactions are primarily surface phenomena it follows within the concept, that an agglutinating serum can be diluted many thousand times as highly before losing the ability to yield a visible agglutination, as it can be before losing the ability to yield a visible precipitate with an antigen dispersed in the form of very small units.

The experimental use of carmine particles as the possible nuclei of fabricated, non-soluble units of antigen would seem to be worthy of trial in the fields of viral infections, allergic disorders and other diseases wherein it is now difficult or impossible to demonstrate antibody mechanisms by conventional methods. In this connection, Smorodintzeff and Fradkina[4] have recently reported their success in using dye particles (carmine) for the adsorption of an antigen and of an antibody concerned in typhus fever.

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[5] Jones, F. S., *J. Exp. Med.*, 1928, **48**, 183.

[6] Zinsser, H., *J. Immunol.*, 1930, **18**, 483.

Summary. By adsorption of equine serum (antigen) to suitable particles of carmine a suspension can be made which is specifically agglutinable in rabbit antihorse serum. With suitably prepared suspensions, the open slide method may be conveniently employed in making agglutination tests. Employing this technic it is possible to detect antibody in the fresh sera of immunized rabbits 2 to 6 days earlier than it is by the conventional precipitation test employing the antigen dilution method.

15003 P

Growth-Promoting and Growth-Inhibiting Factors in Rat Tumor Tissue.*

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The evidence as it exists today supports the view that cell growth-promoting properties of various tissue extracts cannot be correlated with the state of proliferation of the tissues from which they are made.[1] The generally accepted opinion that extracts of actively growing tumors display growth-promoting power to an exceptionally high degree, has also been shown to be erroneous. Tumor extracts appear to affect the rate of cell multiplication *in vitro* to the same extent as extracts of the respective normal tissues. In those species in which normal tissue extracts stimulated growth, tumor extracts acted likewise (*i.e.,* chicken, dog); in the species in which extracts of normal tissues did not affect cell proliferation the tumor extracts are also without growth-promoting power.[2]

The rat is a striking example of a species whose tissues are devoid of cell growth-promoting properties. Apart from extracts of brain, all remaining rat organs and tissues tested are ineffective in activating cell growth *in vitro*.[3] Rat tumors also display no growth-activating effect and even inhibit the proliferation of cell colonies.[2] The lack of growth-promoting power in extracts of normal and neoplastic rat tissues can be explained by either of the following assumptions: (a) Growth-promoting principles are absent in tissues of rats. (b) They are present but their activity is counteracted by coexisting inhibitors. The experiments reported below show that the absence of growth-promoting ability in extracts of rat tumors can be explained in accordance with the second hypothesis.

Experimental. In this study the procedure previously reported for the partial purification of the active principle from growth-promoting tissue extracts was used.[4] The experiments were performed with a benzopyrene sarcoma of rats, extracts of which have a definitely inhibiting effect on the growth of fibroblast colonies *in vitro*.[2] Tumor tissue was finely minced in the Latapie apparatus and the pulp obtained was extracted with 4 volumes of Ringer's solution. The tissue extract was then

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* Aided by a grant from the Dazian Foundation for Medical Research, New York.

† Working under the Cancer Laboratories Fellowship.

[1] Towell, O. A., and Willmer, E. N., *J. Exp. Biol.*, 1939, **16**, 60; Doljanski, L., and Hoffman, R., *C. R. Soc. Biol.*, 1939, **130**, 1246; Hoffman, R., Tenenbaum, E., and Doljanski, L., *Nature*, 1939, **143**, 764; Hoffman, R., and Doljanski, L., *Growth*, 1939, **3**, 61; Hoffman, R., Tenenbaum, E., and Doljanski, L., *Growth*, 1940, **4**, 207.

[2] Doljanski, L., Hoffman, R., and Tenenbaum, E., *Growth*, 1944, **8**, 13.

[3] Hoffman, R., *Growth*, 1940, **4**, 361.

[4] Werner, H., and Doljanski, L., *Nature*, 1942, **150**, 660.

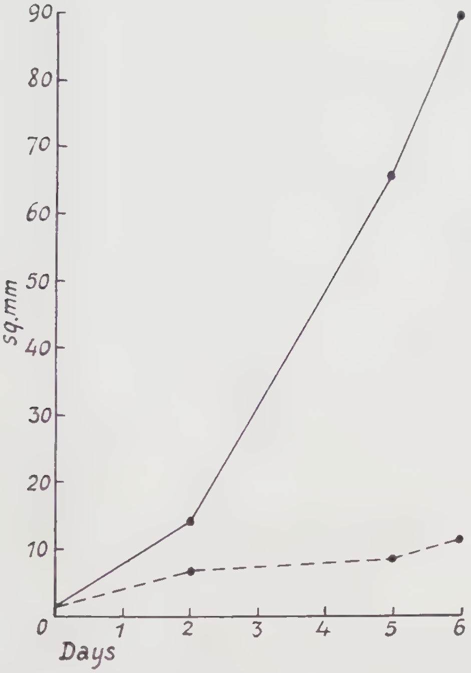

Fig. 1.

Experiment 11220/1 B—Growth curves of sister halves of fibroblast culture in protective medium and in medium containing solutions of alcohol precipitates treated with lipid solvents (acetone and petroleum ether).

———— Alcohol precipitates treated with lipid solvents.

-------- Protective medium.

precipitated with 4 volumes of 96% alcohol. The precipitate obtained was treated successively for 3 hours in a Soxhlet apparatus with acetone and petroleum-ether. After drying *in vacuo* the extracted powder was suspended in Tyrode solution. The suspension was kept for 3 days in the refrigerator, centrifuged and the supernatant fluid was added to standardized cultures of chicken fibroblasts in Carrel flasks. The growth of the cell colonies in medium containing this solution as fluid phase was compared with the growth of controls (sister halves), growing in protective medium composed of plasma diluted with Tyrode in the proportions: 1:2. The growth of cultures was followed over a period of 6 days and recorded according to the method of Ebeling.

The curves of Fig. 1 show the growth of a culture stimulated with alcohol precipitate treated with lipid solvents (acetone and petroleum-ether), compared with that of a control in plasma-Tyrode medium. These curves illustrate the optimal activation obtained; the average stimulation amounted to 520% (13 experiments).

Summary. The experiments reported above indicate that in extracts of rat tumors the activity of the growth-stimulating principle is counteracted by an antagonistic factor. By precipitation with alcohol and subsequent treatment with lipid solvents (acetone and petroleum-ether) it is possible to remove the latter and to convert the originally inhibiting material into a definitely stimulating preparation.

15004 P

The Effect of X-Rays on the Mitotic Activity of Explanted Bone Marrow.*

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The effect of X-rays on the mitotic activity of the bone-marrow has scarcely been studied. In his recent extensive review on the effect of X-rays on bone-marrow, Dunlap[1] mentions the findings of Mottram[2] who observed a

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* This investigation was aided by a grant from the Lady Tata Memorial Trust, London.

† Working on a grant from Mrs. J. H. Stodel, Cape Town, South Africa.

[1] Dunlap, C. E., *Arch. Path.*, 1942, **34**, 562.

[2] Mottram, J. C., *Arch. Radiol. and Electroth.*, 1920, **25**, 197 (quoted by Dunlap[1]).

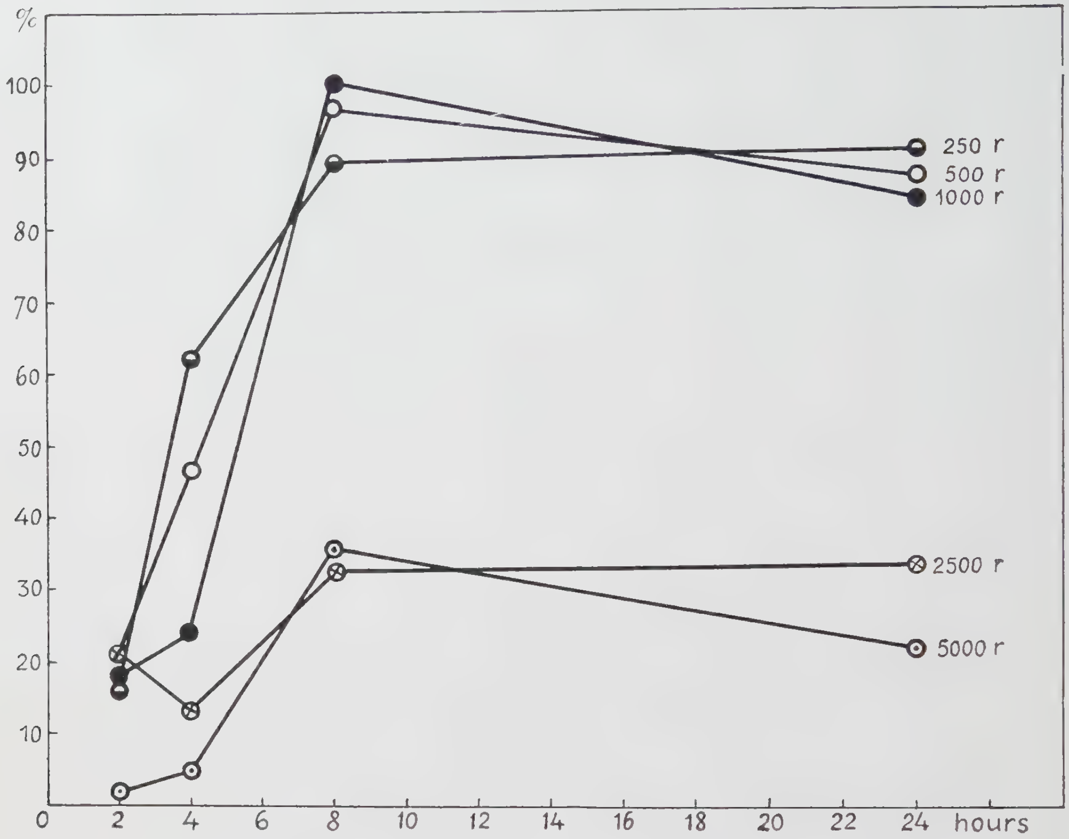

Fig. 1.

Effect of X-rays on Mitoses in Bone-marrow Cultures.

Abscissæ—Time after irradiation in hours.

Ordinate—Number of mitoses in irradiated cultures in percents of number of mitoses in non-irradiated controls.

depression of the mitotic activity in the marrow 2 days after irradiation. Hsü and Ma[3] report that after irradiation of rat bone-marrow with doses of 2500 and 5000 r units mitoses are rare or entirely absent, but they are present in bone-marrow treated with 1000 r units. Osgood and Bracher[4] found a decrease in mitoses after irradiation of suspensions of human bone-marrow cells *in vitro*, but no figures are given to substantiate this observation.

Methods. The present report deals with the mitotic behavior of bone-marrow cultures x-rayed *in vitro*. Experiments were performed on rabbit's bone-marrow cultured in plasma according to the method described elsewhere.[5] Before planting, the bone-marrow was irradiated with the following x-ray doses: 250, 500, 1000, 2500, 5000 r units. The irradiated cultures as well as the non-irradiated controls were incubated for 2, 4, 8, and 24 hours. After incubation the cultures were fixed in Zenker's solution, embedded in paraffin-celloidin, cut in 4 μ sections, and stained with hematoxylin-eosin and Giemsa's stain. The mitotic activity of the bone-marrow cultures was estimated by counting the number of mitoses in irradiated and control cultures respectively, the number of mitoses present in 10 high-power fields being determined in each culture.

Results. All doses applied provoked profound decrease in the number of mitoses in marrow cells 2 hours after irradiation. The number of mitoses in the cultures irradiated with the doses of 250-2500 r units was reduced to approximately 20% of that in non-irradiated controls. In cultures irradiated with 5000 r units the mitoses disappeared almost completely at this time.

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[3] Hsü, C. L., and Ma, W. C., *Am. J. Cancer*, 1940, **39**, 319.

[4] Osgood, E., and Bracher, G. J., *Ann. Int. Med.*, 1939, **13**, 563.

[5] Rachmilewitz, M., and Rosin, A., *Am. J. Med. Sci.*, 1943, **206**, 17.

There was a definite recovery of the mitotic activity 4 hours after irradiation in cultures treated with 250 and 500 r units. The process of the recovery in cultures irradiated with these doses continued and the mitoses reached 8 hours after irradiation values equal to those of the controls. Cultures irradiated with 1000 r units showed no significant recovery after 4 hours, but only after 8 hours; in this case also the recovery was complete. After 24 hours the culture irradiated with 1000 r units and less showed an abundance of mitoses and normal cellularity.

A distinctly different behavior was observed in cultures irradiated with 2500 and 5000 r units. In cultures treated with these doses the recovery took place 8 hours after irradiation; it was incomplete, the number of mitoses rising only to 30% of the values in controls. No further increase of the mitotic count could be observed 24 hours after irradiation. In contrast to the cultures treated with doses of 1000 r units and less, the explanted bone-marrow fragments irradiated with 2500 and 5000 r units, presented after 24 hours a picture of complete or almost complete aplasia.

The mitotic behavior of irradiated bone-marrow cultures in relation to the control cultures is represented in Fig. 1.

Summary. The effect of direct irradiation with X-rays on mitoses in cultures of rabbit's bone-marrow was investigated. The X-rays in all doses applied (250, 500, 1000, 2500, 5000 r units) produced at first a profound decrease in the number of mitoses. In cultures irradiated with doses of 1000 r units and less the decrease in mitoses was followed by complete recovery of the mitotic activity. The recovery of mitoses in cultures irradiated with 2500 r units and more was only incomplete and the irradiation resulted in aplasia of the bone marrow.

15005

Effect of Drop Size and Wall Solution Concentration on Determinations Made with the Hill-Baldes Method.

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The Hill-Baldes vapor tension method of determining osmotic pressure has been used extensively for determining the osmotic activity of biological fluids. This method involves a comparison of the rates of evaporation from or condensation on drops of the sample and of a reference solution when placed on opposite junctions of a thermocouple enclosed in a chamber whose walls are lined with moistened filter paper. The deflection of the galvanometer to which the thermocouple is connected is a measure of the difference in vapor pressure and therefore of osmotic pressure of the two solutions, and is calibrated by using 2 known or reference solutions.[1]

During a recent investigation of the osmotic activity of gastrointestinal fluids following the ingestion of distilled water,[2] osmotic activity readings were made at intervals over a period of from one to 3 days following drop deposition on the thermocouple loops, whereas ordinarily readings are completed within one hour of drop deposition. In the course of these experiments two unexpected difficulties arose: (1) In some cases the apparent osmotic activity of salt solutions fell to values below those of the reference solutions with which they were being compared, and (2) duplicate samples failed to give consistently identical osmotic activity readings throughout the course of a single experiment.

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[1] Roepke, R. R., and Baldes, E. J., *J. Cell. and Comp. Physiol.*, 1942, **20**, 71.

[2] Follansbee, R., *Am. J. Physiol.*, in press.

It appeared probable that factors, control of which may be safely neglected over shorter time intervals, were operating to produce these anomalous results in the case of the longer time intervals. From a consideration of the theory of the method,[3] it is seen that (1) the size of the drops and (2) the difference in osmotic concentration between the drop solution and that on the walls of the thermocouple chamber are such factors. The drops on the thermocouple junctions and the solution lining the chamber wall are continuously approaching diffusion equilibrium with one another by exchange of water. Since the volumes and surfaces of the drops are negligible compared with those of the wall solution each drop may be considered independent of the other and the water exchange to be dependent solely upon the rate at which the drops approach the concentration of the wall solution.

When the wall solution is lower in osmotic activity than the drop the latter will be progressively diluted with time and *vice versa* with the opposite condition. Other conditions being constant, drop size and concentration difference between drop and wall solution determine the rate of equilibration, the smaller the drop the greater the per cent rate of equilibration, and the larger the concentration difference the greater the change in osmotic activity for a given degree of approach toward equilibrium. If the reference and sample drops are of unequal size, they will become diluted at different rates. Since the osmotic activity reading at any given time is a measure of the difference between the two drops at that time, such readings will not represent the osmotic activity differences between the drops at the time of drop deposition, if unequal rates of dilution occurred.

It seemed therefore advisable, in an attempt to explain the difficulties mentioned above, to perform experiments to test the effect on the osmotic activity readings of the concentration of the solution on the walls of the thermocouple chamber and of the size of the drops. The present paper reports the results of these experiments.

Procedures and Results. The apparatus and technic employed were essentially the same as those described by Baldes and Johnson.[4] Four pairs of thermocouples are available in a single chamber and simultaneous duplicate determinations were made in each experiment. Two micrometer burettes[5] with needles attached were set up vertically so that drops of known volume could be placed on the thermocouple loops. Drops containing 0.0012 cc were designated as "small" drops and those containing 0.0032 cc as "large" drops. The experiments were done with known concentrations of sodium chloride in water.

Fig. 1 presents a comparison of duplicate determinations of the rates of equilibration of small and large drops. In this case the sample solution (240 mM NaCl) was the only solution changing concentration with time since both the reference and wall solutions were the same (220 mM NaCl). It will be noted that in accordance with expectations the "large" drops (Fig. 1, Curve I) equilibrated more slowly (about 25% equilibration reached in 30 hours), than did the "small" drop (Fig. 1, Curve II) which came to complete equilibrium in 30 hours or less. It is thus apparent that unless drop size is controlled, large discrepancies between duplicate determinations will occur.

Fig. 2 shows a similar experiment except that the wall solution was 160 mM NaCl. In this case the results are also in accordance with expectations. Since the wall solution was more dilute than either reference or sample drops, both of the latter are approaching the wall solution in concentration, but at different rates due to different drop size. When the sample (240 mM NaCl) drop was

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[3] Baldes, E. J., *Biodynamica*, 1939, **46**, 1.

[4] Baldes, E. J., and Johnson, A. F., *Biodynamica*, 1939, **47**, 1.

[5] Dean, R. B., and Fetcher, E. S., Jr., *Science*, 1942, **96**, 237.

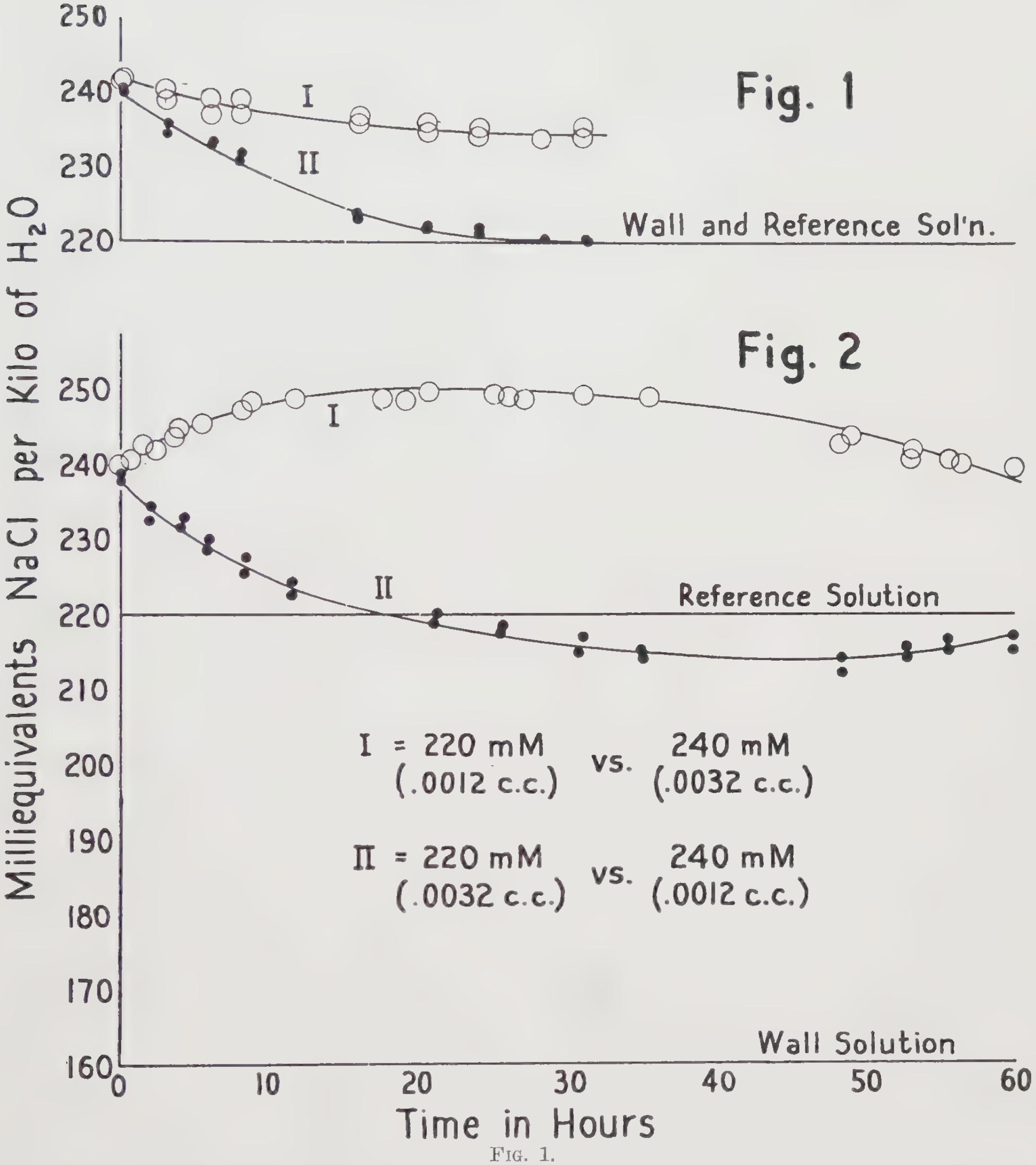

FIG. 1.

Influence of drop size on the osmotic pressure readings when the wall and reference solutions are identical.

FIG. 2.

Artificial osmotic activity readings observed when the unknown and reference drops are of unequal size and the wall solution is more dilute than the reference solution.

large compared to the reference drop (220 mM NaCl, Fig. 2, Curve I) the sample was diluted more slowly than the reference drop so that the concentration difference between the 2 increased for a time. This was read as an increase in osmotic activity of the sample drop. However, when the reference drop was the "large" one, it was diluted more slowly than the sample drop, so that the difference in concentration decreased quite rapidly. This was read as a decrease in osmotic activity (Fig. 2, Curve II). The "small" drop, in this case 240 mM NaCl, was diluted so much faster than the "large" drop that after eighteen hours the 240 mM drop had become more dilute than the 220 mM drop. This was read as a fall below the reference solution.

It is noted that in both experiments described above (Fig. 1 and 2) the initial readings gave correct osmotic pressure values. This is in agreement with Roepke's findings[6] on the effect of drop size on osmotic pressure determinations made with the Hill-Baldes vapor tension apparatus. He states that "it is not necessary under ordinary conditions with dilute solutions that the drops be of exactly the same size." The experiments described in the present report indicate that precautions regarding equal drop size and identical reference and wall solutions must be taken if osmotic pressure readings are to be made over long periods of time after drop deposition on the thermocouple loops. Under some conditions it may be advantageous to control these factors even when the time intervals involved are short.[7] It is interesting to note that the influence of these variables is exhibited in an exaggerated way when the drops consist of a mixture of deuterium oxide and water.[8]

Summary. Experiments are described that test the effect on osmotic pressure readings made with the Hill-Baldes vapor tension method, of the size of the drops placed on the thermocouple loops and of the concentration of the solution placed on the walls of the thermocouple chamber. Evidence is presented indicating that when readings are made over long periods of time after drop deposition on the thermocouple loops the drops must be of equal size and the reference and wall solutions must be identical if accurate osmotic pressure values are to be obtained throughout the course of the experiment.

Acknowledgment is made to Dr. Maurice B. Visscher and Dr. Nathan Lifson for suggestions and assistance in this investigation.

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[6] Roepke, R. R., *J. Phys. Chem.*, 1942, **46**, 359.

[7] Lifson, N., and Lorber, V., *J. Biol. Chem.*, 1945, **158**, 209.

[8] Lifson, N., Lorber, V., and Hill, E. L., *J. Biol. Chem.*, 1945, **158**, 219.

15006 P

The Cellular Transfer of Cutaneous Hypersensitivity to Tuberculin.

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In studies on experimental drug allergy, it has been found that specific hypersensitiveness of the "delayed type" is transferable to normal guinea pigs by means of cells in exudates recovered from sensitized guinea pigs.[1] Resemblances between the delayed type of reaction to drugs and the classical tuberculin reaction have prompted investigation as to whether cells from tuberculin-sensitive animals may likewise transfer tuberculin sensitivity. The experiments show that guinea pigs receiving such cells acquire for a limited time a skin hypersensitivity that exhibits the essential features of the typical tuberculin reaction.

Guinea pigs were rendered hypersensitive to tuberculin by subcutaneous injection of killed human tubercle bacilli suspended in paraffin oil,[2,3] usually mixed with vaseline; each animal received 0.5 to 2.5 mg of dried tubercle bacilli in a total inoculum of 1 cc. Between 5 and 9 weeks later, the cutaneous reactivity to tuberculin then being pronounced, exudates were induced by the intraperitoneal injection of about 28 cc paraffin oil into each of a group of guinea pigs so sensitized. After 48 hours, the peritoneal cavities were washed out with heparinized Tyrode solution containing gelatin or normal guinea pig serum. The washings were combined and the cells recovered from the aqueous layer by minimal centrifugation. The sedimented cells were resuspended in fresh washing fluid by gentle pipetting, and again spun down. A similar washing was made, using Tyrode solution mixed with 1/10 volume of normal guinea pig serum. The washed cells were then suspended in serum-Tyrode and immediately injected into male albino guinea pigs. The yield of cells amounted to 0.1 to 0.15 cc per donor, and recipients were usually given the cells of between 2 and 10 donors. The cells were comprised of 15 to 30% of polymorphonuclear leucocytes, 20 to 35% of lymphocytes, and 50 to 65% of large mononuclear cells. The recipient animals were tested with Old Tuberculin, preferably freed of glycerine by rapid precipitation with cold alcohol, or special preparations containing tuberculoproteins of either the larger or smaller molecular variety;* these were employed in the highest concentration that produced no reactions, or only trivial ones, in untreated control animals.

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[1] Landsteiner, K., and Chase, M. W., PROC. SOC. EXP. BIOL. AND MED., 1942, **49**, 688.

[2] Saenz, A., *C. R. Soc. Biol.*, 1935, **120**, 870, 1050; 1937, **125**, 714; *Ann. Inst. Pasteur*, 1938, **60**, 58.

[3] Freund, J., Casals-Ariet, J., and Hosmer, E. P., PROC. SOC. EXP. BIOL. AND MED., 1937, **37**, 509; Freund, J., Casals-Ariet, J., and Genghof, D. S., *J. Immunol.*, 1940, **38**, 67; Freund, J., and Gottschall, R. Y., *Arch. Path.*, 1942, **34**, 73.

The latent period preceding the development of skin hypersensitivity varies according to the route of administration of the cells—2 to 3 days after intraperitoneal injection, and 20 to 36 hours after intravenous injection.

By way of illustration, in one experiment the cells of 9 sensitized donors were given intraperitoneally to a normal guinea pig, and the first test injections were made 2 hours later with 0.1 cc of 1:4 dilution of "deglycerinated" Old Tuberculin and with "deglycerinated" control broth in like amount; the testing was repeated on each succeeding day. Tuberculin hypersensitiveness became established in the cell recipient in about 48 hours, and exhibited maximal reactivity 72 to 96 hours after injection of the cells. The reaction occurring at the site of the second test injection of tuberculin developed thus:

24 hours: 8 mm (in diameter) faint pink; 3 mm white center; outer diffuse color.

48 hours: 13 mm pale pink, diffuse margin; moderately thickened; 8 mm livid area with 4 mm white center.

72 hours: 16 x 12 mm diffuse faint pink; indurated; 9 mm white center with spotty pink margin; 4 mm brownish necrotic central zone.

The reaction 48 hours after the third test injection with tuberculin was somewhat weaker than that developed during the same interval by the second test injection. After the 5th day, the control animals exhibited an active sensitization to the tuberculin preparation—largely a sensitivity to the broth medium—and the experiment was terminated. This effect is readily differentiable from the typical tuberculin reaction, and can be attained regularly by injecting control broth into normal guinea pigs for 4 to 6 days. Necrosis and sloughing of the test sites occurred in the cell recipient only.

In contrast to the reactions following upon the injection of washed cells, similar effects have not been obtainable with serum of the donors of active cells, or with cells from normal animals.

Of 17 experiments of the sort described, successful transfers have been obtained in 16 instances. The intensity of the transferred hypersensitiveness has varied in accordance with several factors—chiefly the amount of cells used, and the degree of sensitivity of the cell donors.

It has been established that the cells become largely or entirely inactive upon being heated at 48°C for 15 minutes or upon freezing, and are markedly less active after being kept in the icebox over night. The duration of the transferred sensitivity appears to be brief. In addition to exudate cells from the peritoneal cavity, cells from the spleen or lymph nodes are capable of transferring hypersensitiveness to Old Tuberculin. The brief duration of sensitivity and the activity of cells from peritoneal exudates, spleen, and lymph nodes parallel our experiences in the transfer of drug allergy.

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* For these preparations, some of which were freed from constituents of the culture medium, the author is greatly indebted to Dr. Florence B. Seibert.

15007

Further Isolation of St. Louis Encephalitis Virus; Congenital Transfer of Virus in Chicken Mites (*Dermanyssus gallinae*).*

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In a previous publication[1] the isolation of the St. Louis encephalitis virus from chicken mites (*Dermanyssus gallinæ*) was reported. At that time the virus had been demonstrated on two occasions from separate collections of mites from a single chicken house in St. Louis County. Since our earlier report 3 further isolations of the virus have been accomplished from mites obtained from the same chicken house. Collections of chicken mites have also been made from 4 other localities in St. Louis County, and 2 of these have yielded the St. Louis virus. In addition, evidence of the congenital transfer of the virus in chicken mites has been secured.

On 8-20-44, 8-21-44, and 8-22-44 mites, some of which were freshly engorged with blood, were collected from the chicken house which had been the previous source of mites containing the St. Louis virus. The mites were identified and stored in glass tubes at the time of collection and on 8-22-44, 56 of these mites were triturated in an agate mortar with a small amount of tryptose phosphate broth. One-tenth of a ml of this inoculum was given intraperitoneally to each of 7 10-day-old mice. Two of these mice were killed when they showed signs of illness on the seventh day after inoculation. Three other mice developed convulsions on the eighth and ninth days. The brains of the latter 3 mice were stored for future passage and identification of the infective agent. The brain of each of the 2 mice killed on the seventh day after inoculation was emulsified and diluted 1:10 in broth. After centrifugation each supernatant fluid was inoculated intracerebrally into 4 adult Swiss mice. All the mice injected intracerebrally with these inocula developed convulsions in 3 to 5 days.

On 8-29-44 and 9-7-44 mites were collected again from the same chicken house and stored in tubes. On 9-8-44, 78 of these mites were triturated in the manner described above and 8 ten-day-old mice were inoculated intraperitoneally with the mite suspension. Six of the 8 mice developed convulsions in 7 to 10 days. Intracerebral passage of brain tissue of each of the 6 to adult Swiss mice produced convulsions in 3 to 5 days.

Another collection from the above chicken house was made on 10-25-44. In this instance each engorged adult mite was placed in a separate glass tube to be observed for egg laying. Eggs were noted in most of the tubes. These eggs hatched into larvæ which metamorphosed into first stage nymphs within 24 to 30 hours. On 11-3-44 mothers from this collection were separated from their nymphs and were triturated in tryptose phosphate broth in the usual manner. Four nine-day-old mice and 2 eleven-day-old mice were inoculated intraperitoneally with .1 ml of the resultant suspension. Two of the 9-day-old mice developed convulsions on the fifteenth and sixteenth days following the intraperitoneal inoculation, and 1 of the eleven-day-old mice on the fifteenth day. From the brain of each an infective agent was recovered readily and was established in adult mice by intracerebral passage.

Similarly, 147 of the unfed first stage nymphs hatched in tubes from eggs laid by these mothers were triturated in broth and the resultant suspension inoculated intraperitoneally into 5 eight-day-old mice. Two of the 5 showed signs of illness with twitching on the thirteenth and fifteenth days. As before, an infective agent was recovered by secondary intracerebral passage and was

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* Aided by a grant from The National Foundation for Infantile Paralysis, Inc.

[1] Smith, M. G., Blattner, R. J., and Heys, F. M., *Science*, 1944, **100**, 362.

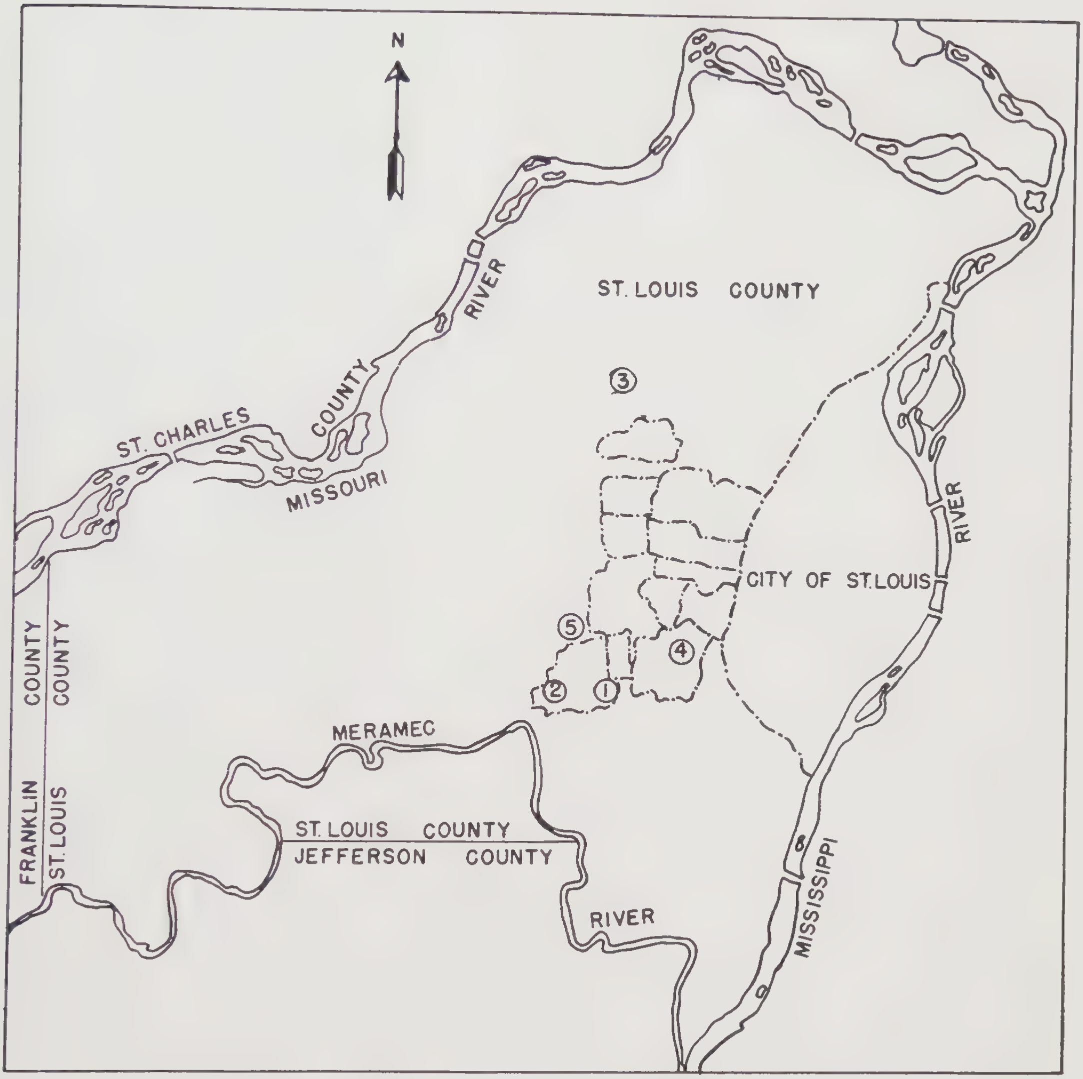

FIG. 1.
1, 2, 3—Sites from which St. Louis virus was isolated.
4, 5 —Sites from which no virus was isolated.

established in adult mice. These results indicate that the infective agent was transmitted congenitally through the egg to the larval stage and through metamorphosis to the first stage nymphs. It should be emphasized that these nymphs were kept in glass tubes from the time that the eggs were laid and were unfed.†

The second chicken house (Fig. 1) from which the St. Louis virus was isolated is approximately 2 miles distant from the site of the first isolation. On 8-18-44 mites, some freshly engorged with blood, were collected and stored. On 8-23-44, 60 of these mites were triturated in tryptose phosphate broth and 4 eight-day-old mice were inoculated intraperitoneally with the mite suspension. Three of the 4 mice developed convulsions on the sixth day following intraperitoneal inoculation. Secondary passage of the infective agent to adult Swiss mice by the intracerebral route produced convulsions in mice in 3 to 4 days.

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† In *Dermanyssus gallinæ* development from larva to the first stage nymph takes place without feeding.

The third site from which mites containing the St. Louis encephalitis virus were collected is approximately 11 miles distant from the second chicken house and 10 miles distant from the original site of isolation. On 9-8-44, 72 mites which had been collected from this chicken house on 9-7-44 were triturated in broth. The suspension was injected intraperitoneally into 4 nine-day-old mice and into 3 eleven-day-old mice. In the case of the nine-day-old mice 3 developed convulsions on the seventh, ninth, and eleventh days following the intraperitoneal inoculation. The infective agent recovered from the brain of each of these mice was carried through 2 intracerebral passages in adult mice. One of the eleven-day-old mice receiving the mite suspension developed convulsions on the tenth day. The infective agent was passed intracerebrally to adult mice as before, producing convulsions in 3 to 6 days.

Routine cultures of the mouse brains in all instances were shown to be bacteriologically sterile. The infective agent isolated in each of the 5 instances was identified as the St. Louis encephalitis virus by means of mouse protection tests using immune St. Louis encephalitis serum. Immune sera were produced by the repeated inoculation of rabbits with 2 of the newly isolated strains. One of these 2 strains was isolated from site No. 1 and the other from site No. 2 (Fig. 1). The stock St. Louis virus (Hibbard strains) was neutralized by each of these 2 immune sera. Thus, the virus of St. Louis encephalitis has been isolated from chicken mites collected from one chicken house on 5 separate occasions, including the 2 previously reported[1] and from each of 2 additional chicken houses. The relative location of these sites is indicated on the map.

The 3 additional isolations (8-22-44, 9-8-44, and 10-25-44) of the St. Louis encephalitis virus from one chicken house 6, 8, and 16 weeks after the initial isolation indicate the persistence of the virus in this population of chicken mites.

A colony established with mites collected from the above chicken house, site No. 1, has been maintained in the laboratory by frequent feeding on normal chickens from 10-5-44 to the present time, a period of 7 months. Two samples of this mite population were tested for the presence of virus on 3-5-45, 5 months after the colony was established, and from both samples the virus was recovered readily by the intraperitoneal inoculation of young mice.

The isolation of the St. Louis encephalitis virus from adult females and also from their unfed first stage nymphs indicates the congenital transmission of the virus in the chicken mite, *Dermanyssus gallinæ*, and offers a plausible explanation for the persistence of the virus in mite populations, both in nature and under experimental conditions.

Summary. The virus of St. Louis encephalitis has been isolated from chicken mites (*Dermanyssus gallinæ*) on 7 separate occasions from 3 of 5 sources in St. Louis County from which mites were collected. Two of these isolations from a single chicken house were reported in a previous publication. Since that time 3 further isolations have been accomplished from mites collected at this original site, and in addition one isolation has been made from each of 2 other chicken houses. All 3 sites are located in St. Louis County within a radius of 11 miles. Mites infected in nature have been shown to retain the virus after 5 months of propagation in the laboratory. Congenital transfer of the virus from the adult female through the egg into the first stage nymph has been demonstrated.

15008

On the Alleged Antitoxic Action of Kombucha for Vitamin D*

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Because of the occasional observation of toxicity from vitamin D, even in comparatively moderate dosage, it would be highly desirable to have available a definite antagonist for use when for any reason it is desirable to continue administration of the vitamin at a given level. Numerous agents have been utilized with varying degrees of success, but none have seemed to be sufficiently dependable for general application.

In a number of publications, Hermann[1] reported that cats were protected against the intoxication induced by high doses of vitamin D as vigantol. The evidence was based on survival time, blood nitrogen level, and on histological examination of the liver, spleen, kidney, aorta, and lung.

This author has described in considerable detail the composition and other characteristics of Kombucha, according to which it is a fungus used in the far east to antagonize the tissue changes incident to aging. An extract of a culture consisted primarily of *d*-gluconic acid, with lesser quantities of keto-gluconic acid, vitamin B-complex, vitamin C, and unspecified enzymes. The *d*-gluconic acid appeared to be the active agent when each component was tested alone. However, the author postulated that the pharmacological effects might be due to the most abundant member, to some unidentified trace substance, or to a combined influence of all the observed constituents. His reports seemed sufficiently conclusive to justify a trial as an anti-intoxicant in rats treated with other forms of vitamin D, since the particular preparation used by Hermann has been claimed to be more toxic than many others. The Kombucha was supplied in generous quantities by Hermann, after his own formula, which information was not supplied in detail to the authors.

Albino rats weighing 200-300 g were treated as indicated in the table for a period of 4 weeks. All animals were on a physiologically adequate diet throughout.

In view of the conclusive nature of the results, it was not deemed worthwhile to examine tissues. The animals in Group 1 gained weight in a physiological range. Those in Group 2 also gained within physiological range, but not so rapidly; possibly the Kombucha inhibited the growth rate slightly. But in all groups receiving toxic doses of vitamin D, there was, uniformly, weight loss that was not inhibited by any range of dosage of the alleged protective agent. The spread was about that occurring in Group 3, which received no Kombucha. All premature deaths occurred in the groups receiving the higher protective doses. The fact that the carcasses were eaten by the survivors doubtless inhibited to some degree the rate of loss in the survivors so that their final weights may have been higher, and the weight losses correspondingly less, than would otherwise have been the case. At any rate, the deleterious effects of hypervitaminosis appeared to be of about the same order in all groups receiving the vitamin whether treated or not, and if treated, irrespective of the dose of Kombucha administered.

Conclusion. 1. The claims of anti-vitamin-D-toxication action by an extract of a fungus, Kombucha, have been investigated in albino rats. 2. When administered orally to rats, a dosage of 100,000 units of vitamin D per kilo per day was definitely toxic to all animals. 3. Kombucha, administered as above, was practically without effect over a period of 4 weeks. 4. Kombucha did not protect rats against vitamin D toxication in any dosage when administered isochronously. 5. So far

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* Supported in part by a grant from the Nutrition Research Laboratories.

[1] Hermann, S., *Klin. Woch.*, 1929, **8**, 1752; *Arch. Exp. Path. u. Pharm.*, 1933, **171**, 756; *Biochem. Z.*, 1933, **266**, 418.

TABLE I.

Group	No. rats	Vitamin D, units/kg	Kombucha, cc/kg	Wt change in g
1	2	0	0	+68, +89
2	2	0	10	+45, +65
3	4	100,000	0	—47, —44, —85, —127
4	4	100,000	2	—70, —75, —98, —150
5	4	100,000	5	—85, —90, —90*, —90*
6	5	100,000	10	—72*, —70, —95, —120, —122

* Died during last week and were eaten by cage mates. Weights recorded at end of 3rd week.

as may be judged from this type of experiment, the claim of antagonism of Kombucha to the toxic action of vitamin D was not supported.

15009 P

Supra-Diaphragmatic Section of Vagus Nerves and Gastric Secretion in Patients with Peptic Ulcer.

THOMAS F. THORNTON, EDWARD H. STORER, AND LESTER R. DRAGSTEDT.

From the Department of Surgery of The University of Chicago.

The studies on gastric secretion to be reported in this paper were performed on 19 patients with duodenal ulcers both before and after division of all vagus fibers to the stomach. The nerves were divided just above the diaphragm by a method previously described.[1] Observations have been made on the continuous night secretion of gastric juice, and the secretory response to histamine, caffeine, insulin, and sham feeding.

a. *Continuous Night Secretion.* There are surprisingly few data on the secretion of the empty stomach of ulcer patients in the absence of any known gastric stimulant. The studies embodied in this report were made as follows: At 9:00 p.m. the stomach was emptied and lavaged with an Ewald tube. When the returns were clear, a Levine tube was introduced through the nose into the stomach and continuous suction maintained until 9:00 a.m. with the Wangensteen apparatus. In 9 patients with carcinoma of the stomach an average of 261 cc of secretion was obtained with a free acidity between 0 and 21 clinical units. In 5 patients with carcinoma of the colon an average of 238 cc was obtained with a free acidity between 0 and 18 clinical units. In 6 patients with gall stones an average of 385 cc of secretion was obtained with a free acidity of 0 to 13 clinical units. In contrast to these findings in 19 patients with duodenal ulcer the night secretion averaged 820 cc with a free acidity between 24 and 82 clinical units. After recovery from the operation for division of the vagus nerves, the ulcer patients secreted an average of 415 cc with a free acidity between 0 and 61 clinical units. These observations indicate an abnormally large secretion of gastric juice commonly occurs in the empty stomach of ulcer patients in the absence of any of the usual types of secretory stimuli and that this excessive secretion is neurogenic in origin.

b. *Gastric Secretion in Response to Histamine.* In 19 patients with duodenal ulcers the gastric secretory response to histamine was determined by the usual technic. In each case an abundant flow of gastric juice with high free acidity was obtained and there was no significant difference in either volume or acidity after vagus section.

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[1] Dragstedt, Lester R., and Owens, Frederick M., Jr., PROC. SOC. EXP. BIOL. AND MED., 1943, **53**, 152; Dragstedt, Lester R., Palmer, Walter L., Schafer, Paul W., and Hodges, Paul C., *Gastroenterology*, 1944, **3**, 450.

c. *Gastric Secretion in Response to Caffeine.* Roth, Ivy, and Atkinson[2] have recently reported that patients with peptic ulcer secrete more gastric juice in response to caffeine than do normal individuals. Using the same method we have studied the effect of caffeine on 17 patients with peptic ulcer. One patient failed to respond either before or after operation. A second patient showed a moderate increase in secretion before the vagus section but not afterward. The other 15 patients responded with an increase in volume and free acidity both before and after operation. The stimulation was as marked after operation as before. In general the effect was less than secured by histamine.

d. *Gastric Secretion in Response to Insulin.* The injection of insulin into experimental animals in doses sufficient to produce hypoglycemia causes a secretion of gastric juice. This stimulating effect seems to be due to the hypoglycemia rather than the insulin since it is abolished by the injection of glucose and it is not seen in the diabetic unless hypoglycemia occurs. Twenty units of crystalline insulin were given to 17 patients with peptic ulcer and subsequently fractional gastric analyses were made on samples of secretion removed at 10-minute intervals. Two of the patients did not respond but in the remaining 15 an increase in the volume and free acidity of the gastric juice was obtained. This effect was often delayed as long as an hour and was accompanied by a low blood sugar and hypoglycemic symptoms. The stimulation was not as marked as with histamine but approximated that obtained with caffeine. It was completely abolished after the vagus section except in one patient where a slight but definite stimulation was secured. The findings confirm the prediction of Hollander that the abolition of response to insulin may serve as a physiological test for the completeness of the vagotomy in ulcer patients.

e. *Gastric Secretion in Response to the Sham Meal.* Sham meals were given to 10 ulcer patients before and after vagus section. The tests were performed by having the patients chew various foods without swallowing. Ten-minute fractional samples of gastric juice were withdrawn for a 60-minute period. Only 3 of the 10 patients displayed a definite increase in volume and free acidity of the gastric juice in response to the sham meal before operation. None responded after operation. It is likely that this test would prove more satisfactory with further experience and the use of more palatable food for the sham meal.

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[2] Roth, J. A., Ivy, A. C., and Atkinson, A. J., *J. A. M. A.*, 1944, **126**, 814.

15010 P

Supra-Diaphragmatic Section of the Vagus Nerves and Gastric Motility in Patients with Peptic Ulcer.

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During the past two and one-half years bilateral section of the vagus nerves to the stomach has been performed on 38 patients with duodenal ulcer, gastric ulcer, or gastrojejunal ulcer. The technic of this operation and some of the results have been reported elsewhere.[1] In this paper we wish to make a preliminary report of some observations that have been made on the motility of the stomach in these patients both before and after operation. Some observations have been made on the relation between the contraction of the empty stomach and the sensation of hunger.

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[1] Dragstedt, Lester R., and Owens, Frederick M., Jr., PROC. SOC. EXP. BIOL. AND MED., 1943, **53**, 152; Dragstedt, Lester R., Palmer, Walter L., Schafer, Paul W., and Hodges, Paul C., *Gastroenterology*, 1944, **3**, 450.

Carlson[2] and others studied the effect of bilateral vagotomy on the gastric motility of normal dogs and found that this procedure usually produced a prolonged decrease in the tonus and a diminution in the number of hunger pangs although the individual contractions remained normal in amplitude. Gastric tetanus was rarely seen. To our knowledge the effect of bilateral section of the vagus nerves on the motility of the empty human stomach has not been reported.

Roentgenologists, as a rule, have reported hypertonicity and hypermotility of the stomach in patients with duodenal ulcer. These observations have been made by fluoroscopic examination of the stomach after the administration of a barium meal. Investigators using the balloon technic for studying the motility of the empty stomach have reported constant motility in some patients and complete atony in others.

In the present study, observations were made on the motility of the empty stomach in 8 patients with peptic ulcer, both before and after bilateral section of the vagus nerves. The experiments were performed in the morning after the patients had fasted since the evening meal the day before. The customary balloon technic was employed. Hypertonicity and hypermotility of the stomach were found in 7 patients before operation. In the eighth patient the motility of the stomach appeared less than normal and in this patient there was a long standing marked pyloric obstruction. Following operation in these 7 cases the gastric tonus was decreased, the hunger contractions when present were normal in duration and decreased in amplitude. Hunger periods were decreased in frequency and duration. In the eighth patient, the tonus and hunger motility were increased from the pre-operative level. It appeared within normal limits after operation.

There was but one patient in this series with sufficient introspection to recognize hunger contractions. In the interval of two weeks since the vagus section, this patient has not experienced hunger. In general, however, the patients on whom the vagus section has been performed have reported no alteration in their sensations of hunger or appetite following the operation. Many have volunteered the information that following operation they have been able to eat a large meal without experiencing the sensation of distention which was common before operation.

Conclusion. 1. When examined by the balloon technic, the empty stomach of patients with peptic ulcer usually displays hypertonicity and hypermotility. 2. Bilateral section of the vagus nerves in patients with peptic ulcer decreases the gastric tonus and hunger contractions of the empty stomach.

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[2] Carlson, A. J., *The Control of Hunger in Health and Disease,* The University of Chicago Press, Chicago, Ill.

15011

Infection and Immunity Following the Intracutaneous Inoculation of Scrub Typhus.*

E. J. Bell and Harry Plotz.†

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The question has been raised by some observers as to whether an active immunity in scrub typhus actually occurs. As more knowledge is acquired with the experimental disease, information on this point is accumulating. Workers have observed that when a mouse escapes death following an intraperitoneal inoculation of infectious material, it usually resists infection when challenged subsequently. As no visible evidence of infection may have been observed following the first inoculation it could be argued that the animal was naturally resistant or that it had developed an inapparent infection. This argument would be less valid for the guinea pig which shows evidence of a general infection following an intraperitoneal inoculation by developing fever or by death, or for the rabbit which develops a severe infection following an intraocular inoculation. In the case of the mouse or guinea pig there is an occasional survival following an intraperitoneal inoculation while the rabbit usually survives an intraocular inoculation. Those guinea pigs and rabbits that survive are usually immune and the evidence is accumulating that in the experimental animal at least, an active immunity follows a severe infection.

The experiments which are to be reported bear on this point. The data indicate that a solid immunity in guinea pigs, rabbits, and hamsters can be induced at will following a minimal skin infection.

Rabbits. Six normal rabbits weighing approximately 3 to 4 lb were given 2 intracutaneous inoculations in the abdominal region of 0.1 cc of infected ascitic fluid, diluted 1/20 in broth. The infectious ascitic fluid was obtained from a guinea pig which was sacrificed on the fourth febrile day following the inoculation of the Host 21 mite-strain of scrub typhus. Preparations of the ascitic fluid revealed numerous typical intra- and extra-cellular rickettsiæ.

On the seventh day following inoculation all 6 rabbits showed a small, indurated papule at the site of inoculation. These papules were approximately ½ cm in diameter and slightly raised. They were indurated, with a mild inflammatory reaction about them, and remained so for 2 to 3 days. These lesions did not go on to ulceration, but some showed a small necrotic center and a dry scab. The local lymph glands were not enlarged. These indurated lesions persisted for a period of 6 to 8 days when they gradually regressed and disappeared.

Twenty days following the intracutaneous inoculations, 5 of the rabbits were tested for immunity by the intraocular inoculation of 0.15 cc of infectious mouse blood, diluted 1/30 in saline. Five normal rabbits, similarly inoculated at the same time with the same infectious material, served as controls. The inoculum was obtained from an infected mouse representing the 14th serial passage in mice with the Host 21 mite-strain of scrub typhus. Three mice were also inoculated with this suspension to serve as controls on the infectivity of the inoculum. The three mice died on the 13th day after inoculation showing typical signs of scrub typhus with the characteristic peritoneal exudate containing intra- and extra-cellular rickettsiæ.

All of the 5 control rabbits developed the typical ocular reaction following the intraocular inoculation of infectious material. The average incubation period was 11 days at which time a severe, diffuse conjunctivitis was noted with a circumcorneal injection and a

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[*] Submitted to the Director, U. S. A. Typhus Commission, 24 July, 1944.

[†] Members, United States of America Typhus Commission.

considerable degree of turbidity of the aqueous chamber. Pannus appeared about the 12th day in all animals, which progressed to cover the whole cornea from the 28th to the 30th day following inoculation. Turbidity rapidly progressed to a maximum and persisted for more than 30 days. Active signs of infection were present in all animals for a period of approximately 28 days.

The ocular reactions in the animals that had received the intracutaneous inoculation previously were entirely different. All 5 of the rabbits were observed over a period of 30 days and none developed the slightest degree of infection. These animals were solidly immunized following the intracutaneous inoculation.

Guinea Pigs. Seven normal guinea pigs were given 2 intracutaneous inoculations of 0.1 cc each of infectious ascitic fluid obtained from a guinea pig infected with the Host 21 mite-strain of scrub typhus. The inoculations were made in separate areas in the abdominal region. A small indurated nodule developed at either one or both areas of injection and appeared from the seventh to the tenth day after inoculation. These lesions persisted for from 2 to 6 days. Outside of this small skin lesion no other evidence of infection was observed.

Fifteen days after the intracutaneous inoculation the 7 guinea pigs were challenged by the intraperitoneal inoculation of 1.0 cc of infectious ascitic fluid obtained from a guinea pig reacting to infection with the Host 21 mite-strain. Seven normal guinea pigs were similarly inoculated to serve as controls. Temperatures of all of the guinea pigs were taken daily for 24 days. All of the control guinea pigs showed varying degrees of temperature, which is the characteristic picture in guinea pigs following the inoculation of the strain under study. Only one of the guinea pigs that had previously received the intracutaneous inoculation developed a febrile reaction above 103.4°F on the 6th to the 19th and 20th day. None of the other guinea pigs developed a febrile reaction. The difference in the febrile reactions occurring in the guinea pigs that had received the intracutaneous inoculation before challenge and the controls was clear-cut.

Hamsters. Six hamsters weighing from 100-130 g were inoculated intracutaneously with 0.2 cc of mouse ascitic fluid obtained after infection with Host 22 mite-strain. This exudate contained many intra- and extracellular rickettsiæ. Approximately 0.1 cc of exudate was diluted with 2.0 cc of broth and 0.2 cc was inoculated intracutaneously. All 6 hamsters developed a small indurated papular lesion by the 5th day. These lesions persisted for 3 or 4 days and then regressed. The animals showed no other visible appearance of disease. Thirteen days after the intracutaneous inoculation these 6 hamsters as well as 6 controls were inoculated intraperitoneally with the Kostival yolk sac culture (40th passage). Each animal received 1 cc of a 10% yolk sac suspension. All the control hamsters died in from 11 to 17 days with a typical scrub typhus infection, while all the hamsters that had previously received the intracutaneous inoculation survived. It is worth noting that the intracutaneous inoculation was made with Host 22 mite-strain and that the animals were challenged with the Kostival strain. There was a complete cross-immunity between these two strains.

Conclusion. When rabbits, guinea pigs, and hamsters are inoculated intracutaneously with small doses of infectious peritoneal exudate, an indurated papular lesion develops which lasts a few days and then regresses to disappear completely. We have no evidence on the possible distribution of the agent following the intracutaneous inoculation. When these animals are subsequently challenged by the inoculation of infectious material, they are shown to possess a solid immunity, while the controls show signs of active disease or die. These experiments fit in with observations made by others as well as by us, namely, that an active immunity against scrub typhus in the experimental animal can be induced.

15012

Repository Injection of Penicillin in Water-in-Oil Emulsion. I. Effect on Gonorrhea.[*]

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There have been numerous efforts to improve the administration of penicillin by either prolonging its absorption[1] or delaying its excretion.[2] Most recently methods of administering penicillin by mouth have been reported.[3]

This study was undertaken to determine if penicillin injected intramuscularly is more effective in a water-in-oil emulsion than in aqueous solution. It was hoped that the absorption of penicillin might be prolonged by incorporating it in a water-in-oil emulsion so that the number of injections and perhaps the amount of penicillin could be reduced. Patients with gonococcal infections were chosen for testing the method because clinical manifestations are usually clear-cut and cure can be established readily by bacteriological methods.

Water-in-oil emulsions were prepared by combining an aqueous solution of sodium penicillin with a lanolin-like substance[†] and peanut oil (other vegetable oils may be used). They contain microscopic droplets of aqueous solution of penicillin in a menstruum of oil and "Falba."[†] (Fig. 1.) Such preparations can be made conveniently in a clinical laboratory as follows: One part of autoclaved and melted "Falba" is placed in a mortar and 1.3 parts of a penicillin solution are added to the "Falba" drop by drop while mixing with a pestle. Then 1.8 parts of autoclaved peanut oil are mixed slowly with the penicillin-"Falba" mixture. Solutions containing up to 200,000 units of sodium penicillin per ml can thus be incorporated; it has been found convenient, however, to use solutions containing 100,000 units per 1.4 ml of normal saline. A somewhat simpler procedure, particularly useful in preparing larger amounts of emulsion, is to mix the "Falba" with peanut oil and add the aqueous solution of penicillin slowly to the oil-Falba mixture while it is being agitated by an electric stirrer.

A single dose can be prepared without laboratory facilities in the following way: One and four-tenths ml of sterile 0.85% salt solution are drawn into a sterile syringe and ejected into a vial containing 100,000 Oxford units of dry penicillin. To this penicillin

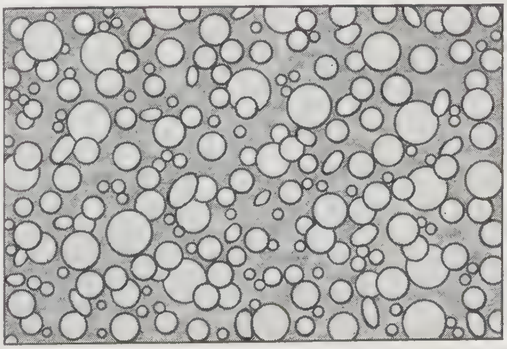

Fig. 1.

Schematic drawing of a water-in-oil emulsion of aqueous penicillin solution in peanut oil. The penicillin is contained in the water droplets.

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[*] The laboratory work was aided by a grant from the U. S. Public Health Service.

[1] (a) Romansky, M. J., and Rittman, G. E., *Science,* 1944, **100**, 107; (b) Trumper, M., and Hutter, A. M., *Science,* 1944, **100**, 432; (c) Fisk, R. T., Foord, A. G., and Alles, G., *Science,* 1945, **101**, 124.

[2] (a) Rammelkamp, C. H., and Bradley, S. E., Proc. Soc. Exp. Biol. and Med., 1943, **53**, 30; (b) Beyer, K. H., Woodward, R., Peters, L., Vermey, W. F., and Mattis, P. A., *Science,* 1944, **100**, 107.

[3] (a) Libby, R. L., *Science,* 1945, **101**, 178; (b) McDermott, W., Bunn, P. A., Benoit, M., Du Bois, R., and Haynes, W., *Science,* 1945, **101**, 228.

[†] The lanolin-like substance used in this study is sold under the trade name of "Falba," manufactured by Pfaltz and Bauer, Inc., New York City.

TABLE I.
Serum Penicillin Concentration in Patients Given a Single Intramuscular Injection of 150,000 Oxford Units of Penicillin.

Patient	Penicillin, Oxford units: Aqueous solution, × 1,000	Penicillin, Oxford units: Water-in-oil emulsion, × 1,000	Hours after injection: ½	1	2	4	5	6	7	8
W	150		1.25	.31	.16	0	—	0	—	0
L	150		2.5	2.5	.31	0	0	0	0	—
F		150	.62	.16	.04	0	—	0	—	0
R		150	1.25	.31	.16	.04	—	0	—	0
Q		150	1.25	1.25	.62	.06	0	0	0	—
P	50	100	.31	.31	.78	.02	—	0	—	0
C	50	100	1.25	1.25	.31	.06	.008	0	.008	0
D	50	100	2.5	1.25	.62	.31	.062	.008	.008	—

0 = No penicillin demonstrable.
— = No sample.

TABLE II.
Duration of Urinary Excretion of Penicillin in Patients Given a Single Intramuscular Injection 150,000 Units of Penicillin

Patient	Penicillin, Oxford units: Aqueous solution, × 1,000	Penicillin, Oxford units: Water-in-oil emulsion, × 1,000	Hours after injection: ½	2	4	5	6	8	10	24	28
W	150		—	—	+	—	+	+	+	0	0
L	150		—	+	+	—	+	—	—	—	—
F		150	+	+	+	—	+	+	+	+	—
R		150	—	—	+	+	—	+	+	—	—
Q		150	—	+	+	—	+	—	—	+	+
P	50	100	—	+	+	+	+	+	+	+	—
C	50	100	—	+	+	—	+	—	—	+	+
D	50	100	—	+	+	—	+	—	—	+	—

— = No sample.
+ = Penicillin present—at least 0.005 unit per ml.
0 = No penicillin demonstrable.

solution 3.1 ml of an autoclaved mixture containing 11 parts of a lanolin-like substance and 20 parts peanut oil are added by means of a sterile syringe and needle of 17 gauge. With the needle still inserted through the rubber cap of the vial, the mixture can be readily emulsified by repeated withdrawals and ejections. As soon as the mixture assumes a uniformly creamy, slightly viscid consistency, satisfactory emulsification has occurred and it is ready for injection.[4]

Serum penicillin levels (Table I) following single intramuscular injections of penicillin in aqueous solution and water-in-oil emulsion were determined by the method of Rammelkamp.[5]

Table II shows the time intervals after injection of penicillin at which penicillin is demonstrable in the urine.

The results of treating patients with gonococcal infection (proved by culture) by means of single injections of penicillin are shown in Table III. The table shows that the simultaneous injection of 100,000 units of penicillin in water-in-oil emulsion and 50,000 units in aqueous solution (Group 6) cured all of 49 consecutive cases. This method of injecting a water-in-oil emulsion combined with an

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[4] Freund, J., and Thomson, K. J., *Science*, 1945, **101**, 468.

[5] Rammelkamp, C. H., PROC. SOC. EXP. BIOL. AND MED., 1942, **51**, 95.

TABLE III.
The Effect of a Single Intramuscular Injection of Penicillin on Acute Gonococcal Infection.

	Penicillin, Oxford units			Males			Females			Total		
Group	Aqueous solution, × 1,000	Water-in-oil emulsion, × 1,000	Total, × 1,000	T.	C.	F.	T.	C.	F.	T.	C.	F.
1	150	0	150	13	11	2	0	0	0	13	11	2
2	300	0	300	8	7	1	0	0	0	8	7	1
3	0	150	150	43	41	2	9	9	0	52	50	2
4	50	50	100	7	6	1	2	2	0	9	8	1
5	50	75	125	10	9	1	3	3	0	13	12	1
6	50	100	150	38	38	0	11	11	0	49	49	0
7	50	150	200	8	8	0	0	0	0	8	8	0

T = Total number of patients treated.
C = Cures. Minimum criteria for cures are: in males at least 3 negative smears and cultures of urethral and prostatic secretions in 21 days; in females, at least 3 negative smears and cultures of urethral and cervical secretions in 21 days.
F = Failures.

aqueous solution as a "priming" dose was reached on the basis of the previous observation showing that 150,000 units in water-in-oil emulsion alone (Group 3) failed to cure all patients so treated. A single injection of 150,000 units in salt solution (Group 1) resulted in 2 failures among 13 cases. A single injection of 300,000 units in salt solution (Group 2) resulted in 1 failure among 8 cases.

Immediate reactions to the intramuscular injection of water-in-oil emulsions either alone (Group 3) or in combination with aqueous solution (Groups 4, 5, 6, 7) were not different from those occurring when aqueous solutions were injected. These consisted chiefly of transient pain at the site of injection radiating down the thigh, often associated with a sensation of "stiffness" of the leg. Such discomfort gradually subsided after 3 to 5 minutes. The site of injection was examined carefully during the follow-up period (up to 3 weeks) and no local infiltration or other abnormalities were found.

Water-in-oil emulsions of antigens such as diphtherial toxoid, horse serum, typhoid and Bordet-Gengou bacilli and gonococci have been injected in animals and man.[6] It was found that antibody formation is sustained for a remarkably long time after the injections of antigens in water-in-oil emulsions. It was also found that mice tolerate the injection of approximately one hundred lethal doses of tetanus toxin when administered in water-in-oil emulsion.[7] These observations suggest that absorption of substances from the aqueous phase of a water-in-oil emulsion is considerably prolonged. Strauch's observation that the absorption of strychnine and morphine is very much retarded from water-in-oil emulsions[8] supports this interpretation.

The present method used for treating gonorrhea with penicillin consists of at least 3 intramuscular injections of a total dosage of 100,000 to 150,000 Oxford units of penicillin during a 4-hour period. The method described here which requires a single injection of penicillin has obvious advantages.

The technic of administering therapeutic agents employing repository injections, affords wide clinical and investigational possibilities not only for penicillin but for other substances as well.

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[7] Freund, J., and Behan, M. A., unpublished experiments, 1941.

[8] Strauch, C. B., *J. Am. Med. Assn.*, 1929, **92**, 1177.

15013

Experiment in Cross Immunity Between Infectious Hepatitis and Homologous Serum Jaundice.*

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Planned experiments with human volunteers have demonstrated similarities in the properties[1-5] of the etiologic agent (or agents) of *infectious hepatitis* and *homologous serum jaundice* in that the agent (or agents) of both conditions is filtrable, resistant to heating to 56°C for 30 minutes, and can be transmitted in serial passage to human volunteers. Comparative studies of both clinical and experimental cases, however, reveal apparent differences between the two conditions. Primarily there is a more or less consistent difference in incubation period with a prolonged period (60-120 days) in homologous jaundice[6-9] in contrast to the shorter period (20-40 days)[10-13] of infectious hepatitis; there may, however, be some overlapping. Secondly, in contrast to the infective character of infectious hepatitis there is usually failure of contact cases to develop in homologous serum jaundice, although exceptions to the latter are recorded.[14-15] Because of these apparent differences and similarities, there is confusion concerning the nature of the two conditions. Homologous serum jaundice may represent the artificial production of infectious hepatitis but this does not seem to be always true. As yet there is no means of clarifying this situation. Findlay together with Martin and Mitchell[15] have suggested that *size of dose, individual susceptibility* and *route of inoculation* may be the factors conditioning the differences.

There is little available information concerning immunity in infectious hepatitis or immunological relationships between these two conditions. Recently Oliphant[16] reported failure to infect 10 human subjects, convalescent 6-19 months from homologous serum jaundice, when inoculated with a strain of

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* Representing work done for the Neurotropic Virus Disease Commission of the Board for the Investigation and Control of Influenza and Other Epidemic Diseases in the Army, in the Preventive Medicine Service of the Office of the Surgeon General, U. S. Army, Washington, D.C.

Acknowledgment is made of the assistance and cooperation of the following agencies: Selective Service, Camp Operations Division; Civilian Public Service Unit No. 81; the Connecticut State Hospital, Middletown, Conn.; Federal Correctional Institution, Danbury, Conn.; and U. S. Public Health Service.

[1] Havens, W. P., Jr., Proc. Soc. Exp. Biol. and Med., 1945, **58**, 203.

[2] Oliphant, J. W., Gilliam, H. G., and Larson, C. L., *Pub. Health Rep.*, 1943, **58**, 1233.

[3] MacCallum, F. O., and Bauer, D. J., *The Lancet*, 1944, **1**, 622.

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[6] Sawyer, W. A., Meyer, K. F., Eaton, M. D., Bauer, J. H., Putnam, Persis, and Schwentker, F. F., *Am. J. Hyg.*, 1944, **39**, 337; **40**, 35.

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[10] Pickles, W. N., *Epidemiology in County Practice*, Balto., Williams and Wilkins Co., 1939, p. 59.

[11] Kligler, I. J., Btesh, D. S., and Koch, W., *J. Inf. Dis.*, 1944, **74**, 234.

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[13] Havens, W. P., Jr., Ward, R., Drill, V., and Paul, J. R., Proc. Soc. Exp. Biol. and Med., 1944, **57**, 206.

[14] Propert, A. S., *Brit. M. J.*, 1938, **2**, 677.

[15] Findlay, G. M., Martin, N. H., and Mitchell, J. B., *The Lancet*, 1944, **2**, 304, 340, 365.

[16] Oliphant, J. W., *Pub. Health Rep.*, 1944, **59**, 1614.

infectious hepatitis which produced jaundice in 4 of 10 normal control subjects, with incubation periods ranging from 85-106 days.

The present report describes the lack of demonstrable immunity in human volunteers, convalescent from homologous serum jaundice when re-infected with a strain of infectious hepatitis.

Strains Employed. Serum jaundice. The strain of homologous serum jaundice was derived from a pool of serum obtained from 11 men in the first 24 hours of sandfly fever in the Middle East. This pool was prepared by Lt. Col. A. B. Sabin, M.C.[17] This icterogenic agent conforms in behavior to other strains of serum jaundice in that out of 23 trial inoculations, 11 cases have been produced, with incubation periods ranging from 60-132 days in both 1st and 2nd passages.[4]

The patients who furnished the pool of serum described above had all been living (during June, 1943) within an area endemic for infectious hepatitis, although at the time the samples were obtained the prevalence of the disease was low. The donors had been questioned specifically at the time the blood was drawn and had stated that they had not had jaundice recently. Subsequent information from 10 of these men, 4 months after bleeding, revealed that none of them had developed jaundice. The 11th man had left the country and could not be traced.

The first transmission experiments, carried out in the Middle East, were originally concerned with sandfly fever.[17] The pool of serum described above produced sandfly fever in 9 out of 10 inoculated human volunteers after the usual incubation period of from 3-5 days. Subsequently, 4 of these 10 volunteers developed jaundice at intervals of 72 to 94 days after inoculation, indicating that the original pool of serum also contained an icterogenic agent.

Several months later this experiment was repeated in the United States inoculating the original pool of serum alone (and mixed with first passage serum).[13] The serum was heated to 56°C for 30 minutes before inoculation to destroy the virus of sandfly fever known to be present. Jaundice was produced in 3 out of 5 human volunteers with incubation periods of 56 to 70 days.

Subsequent test of these 3 men for cross immunity to the naturally occurring disease infectious hepatitis is reported here.

Infectious hepatitis. The virus employed was obtained from a pool of stools and urine of patients with infectious hepatitis hospitalized in the Middle East. It was originally transmitted to 2 human volunteers by feeding with incubation periods of 20 and 22 days.[13] This virus is filtrable, resistant to a temperature of 56°C for 30 minutes[1] and has been through 3 passages in human subjects, producing hepatitis after ingestion or parenteral inoculation of the infectious agent with incubation periods ranging from 16 to 34 days.

The inoculum employed in this experiment was serum obtained during the first 5 days of infectious hepatitis (pre-icteric phase) which had been experimentally induced in 2 human volunteers by feeding feces (and urine) obtained from *naturally occurring* cases. As such it constitutes *first human passage* material. The serum was stored at dry ice box temperature for a period of 2-5 months. Before using, it was thawed at room temperature, pooled, filtered through a Chamberland No. 2 filter and immediately afterward heated to 56°C for 30 minutes.

The heated filtrate of serum was administered in amounts of 1 cc (parenterally or orally) to each of 16 human subjects and the results are described in Table I. These men were residents in a penal institution and a hospital for mental disease, and volunteered for the experiment. There had been no infectious hepatitis in either institution for the preceding year and no other cases have occurred during the period of the experiment.

It is of interest that the 3 men who had previously contracted *homologous serum jaundice* with a long incubation period of 56, 66, and 70 days respectively after parenteral inoculation, when re-inoculated sub-intracutaneously after 6 months of convalescence with a strain of naturally occurring *infectious hepatitis,* all contracted infectious hepatitis with

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[17] Sabin, A. B., Philip, C. B., and Paul, J. R., *J. A. M. A.*, 1944, **125**, 603, 693.

TABLE I.
Results of Administration of Icterogenic Serum (Infectious Hepatitis) to Human Volunteers.

Group	Subjects	Route	Number of volunteers: Inoculated	Number of volunteers: Jaundiced	Incubation period, days
A	Normal subjects	Par.*	7	2	24, 31
B	Inoculated unsuccessfully 6 months before with agent of homologous serum jaundice	”	1	1	21
C	Convalescent 6 months from homologous serum jaundice	”	3	3	20, 20, 25
D	Normal subjects	Oral	4	3	23, 24, 34
E	Fed unsuccessfully 6 months before with agent of homologous serum jaundice	”	1	1	29

* Par. = Parenteral inoculation.

jaundice after 20, 20, and 25 days respectively. It is also of note that the incubation periods in these experimental cases have ranged from 20-34 (average 24) days, with no appreciable difference between those inoculated parenterally and those fed equal amounts of the same infectious agent.

Summary. Previous experimental infection (or inoculation without infection) with a strain of *homologous serum jaundice* obtained in the Middle East failed to protect 5 human volunteers against subsequent experimental infection induced 6 months later with a strain of *infectious hepatitis* also obtained in the Middle East.

These results are in contrast to those found by Oliphant, who has described immunity in human subjects convalescent 6-19 months from homologous serum jaundice, when inoculated with serum from acute cases of infectious hepatitis.

Using a strain of the *naturally occurring disease,* infectious hepatitis, no appreciable difference in incubation period was observed in experimental cases when equal amounts of the same infectious agent were inoculated parenterally or fed. Incubation periods ranged from 20-34 (average 24) days.

15014

Immunologic Relationships of MM, Lansing Poliomyelitis and Mouse Encephalomyelitis Viruses.

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A number of viruses have the physical properties of poliomyelitis virus and induce poliomyelitic lesions in rodents. Those discovered by Theiler are known as mouse encephalomyelitis virus.[1] The others are thought to be strains of human poliomyelitis which have been adapted to rodents.[2-6] Both groups include strains which differ clinically and in infectivity from human poliomyelitis and spontaneous mouse encephalomyelitis.† Largely because of this Jungeblut's suggestion[7] that

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* Present address: Division of Laboratories and Research, New York State Department of Health, Albany 1, New York.

[1] Theiler, M., *J. Exp. Med.*, 1937, **65**, 705; *Medicine*, 1941, **20**, 443.

[2] Armstrong, C., *Pub. Health Rep., U.S.P.H.S.*, 1939, **54**, 1719.

[3] Jungeblut, C. W., and Sanders, M., *J. Exp. Med.*, 1940, **72**, 407.

[4] Toomey, J. A., and Takacs, W. S., PROC. SOC.

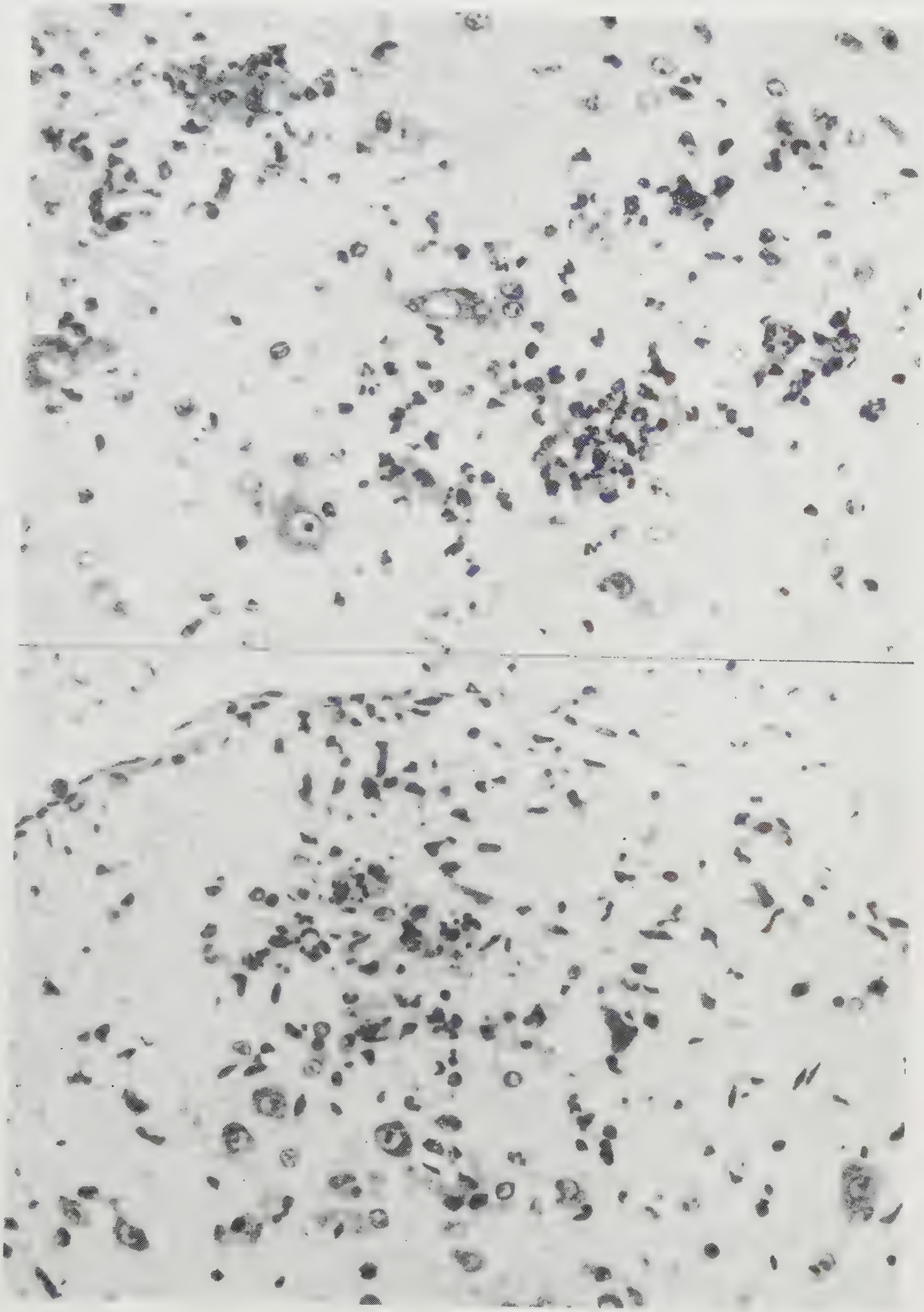

Fig. 1.

Lesions of anterior horns of hamsters following intraperitoneal injection of MM virus. The upper photograph is of the thoracic cord and shows several areas of neuronophagia with polyblasts and polymorphonuclears. The lower photograph shows a minute lesion in the sacral cord of another animal. Both specimens were prepared from hamsters which had been paralyzed for more than a week.

TABLE I.
Neutralization of MM Virus by Various Sera.

A—Mouse Tests
Days on which mice died or were paralyzed
Virus dilutions

Serum	10-2	10-4	10-6	10-8	10-10
MM convalescent hamster	3,5	5,0	0,0	0,0	0,0
GDVII convalescent hamster	2,2	2,3	2,2	2,3	3,3
McG hyperimmune rabbit*	2,2	2,2	2,3	3,3	2,3
Lansing hyperimmune rabbit	3,5	3,4	0,0	0,0	0,0
MM hyperimmune rabbit	3,3	5,5	0,0	0,5	0,0
Normal rabbit	1,2	2,2	3,3	3,3	2,3
Normal hamster	2,2	2,2	2,2	2,3	3,3

* A strain of mouse encephalomyelitis virus (see Table II).
Mixtures injected intracerebrally in 3-week-old Swiss mice (0.03 cc).

B—Hamster Tests
Virus dilutions

	10-3			10-6		
	No. tested	No. paral.	No. died	No. tested	No. paral.	No. died
MM convalescent hamster	6	0	0	6	0	0
Lansing hyperimmune rabbit	6	4	0	6	0	0
Lansing convalescent hamster	6	6	2	6	6	1
Normal rabbit	6	5	4	6	5	1

Mixtures injected intraperitoneally in young animals (0.1 cc).

all be included in a "poliomyelitis group" has not been adopted.

MM virus, apparently derived from a human case of poliomyelitis by hamster passage,[5] is highly infectious and is present in large amounts in diseased animals. It is infective by peripheral as well as central routes. On the other hand the lesions (Fig. 1) seem to us to be typical of poliomyelitis and the physical properties[5] are similar to those of human strains. MM virus therefore renews the problem of terminology and taxonomy and the present report is submitted as a contribution to that problem as well as a further documentation of the MM virus.

Virus Neutralization. The sera of convalescent hamsters effectively neutralize MM virus. The antibodies appear within 10 days and rapidly increase. Sera collected during the 4th week usually neutralize more than a million infective doses.

The sera of hamsters convalescent from Lansing poliomyelitis or weak strains of mouse encephalomyelitis virus do not contain appreciable amounts of antibody but potent antisera can be prepared by repeatedly injecting rabbits with mouse brain suspensions from diseased animals. The sera used in the present work were prepared in this manner. Eight injections were given at 3- or 4-day intervals. The first 4 injections consisted of 5 cc of a 10% suspension of mouse brain injected intraperitoneally while the last 4 included, in addition, 0.5 cc of 0.1% suspensions injected intravenously. The final intravenous injection was of 10% suspension.

Using such sera and incubating equal parts of them and various dilutions of virus for 2 hours at room temperature it has been found that Lansing poliomyelitis hyperimmune rabbit serum neutralizes MM virus very

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EXP. BIOL. AND MED., 1940, **45**, 364; 1941, **46**, 22, 319.

[5] Jungeblut, C. W., and Dalldorf, G., *Am. J. Pub. Health*, 1943, **33**, 169.

[6] Schlesinger, R. W., Morgan, I. M., and Olitsky, P. K., *Science*, 1943, **98**, 452.

[†] Olitsky (Olitsky, P. K., PROC. SOC. EXP. BIOL. AND MED., 1945, **58**, 77) has recently discussed the significance of these differences.

[7] Jungeblut, C. W., *Am. J. Pub. Health*, 1943, **33**, 1227.

TABLE II.
Cross Immunity Tests in Mice Previously Injected Intracerebrally with Mouse Encephalomyelitis Virus (McG Strain).

Test virus	Day on which the mice died or were paralyzed	
	Experimental group	Control group
Theiler's	11,11,12,19,0,0,0	11,11,12,12,13,19,19
(TO 0.03 cc IC)	0,0,0,0,0,0,0,0	23,23,23,29,0,0,0
MM (IP)		
10-1	3,4,5,5,5,6	4,4,4,4,5,7
10-3	4,6,10,10	4,6,9,10
10-5	5,5,0	5,0,0
MM (IC)*		
10-3	7,7	7,7
10-6	7,7	7,0

* Large adult Swiss mice. 10-6 is end point in mice of this age and strain. All groups of same age and strain. All tested mice non-paralyzed convalescents. Interval between injections 28 days.

TABLE III.
Cross Immunity Tests in Hamsters Previously Injected Intraperitoneally with MM Virus and Challenged with Lansing Poliomyelitis Virus Injected Intracerebrally.

Response to MM virus	Number	Response to Lansing virus
Experiment 1. Interval 14 days.		
Paralyzed but completely recovered	18	0 paralyzed
Not paralyzed	18	6 "
Normal controls	19	5 "
Experiment 2. Interval 32 days.		
Paralyzed but completely recovered	7	0 "
Paralyzed. Slight residual paralysis in hind legs*	11	No further paralysis
Not paralyzed	15	0 paralyzed
Normal controls	23	6 "

(In both experiments animals of the same age were used throughout.)
* Lansing poliomyelitis affects forelegs more frequently than hind legs. In 4 of the 6 controls paralysis was limited to the forelegs.

effectively while mouse encephalomyelitis hyperimmune rabbit serum does not (Table I). The latter effectively neutralizes homologous virus in 10% suspensions of mouse brain.

Tests have also been made of these antisera against Lansing virus including trials in which the antisera were mixed with equal amounts of glycerin and the virus containing nervous tissue stored in the mixtures for as long as 3 weeks; the virus content was then determined by mouse inoculation. The number of paralyzed mice can be reduced from 73% to 53% by using either Lansing hyperimmune rabbit serum or MM convalescent hamster or hyperimmune rabbit serum. The mice which receive the test sera rather than normal rabbit serum also exhibit delayed onset of paralysis. These tests have been repeated by Jungeblut, using our sera, with similar results.[8] It is interesting to note that convalescent serum from A.D., one of the patients of the 1942 epidemic from which the MM virus originated, likewise neutralizes Lansing virus to this degree.

Cross Immunity Tests. Cross immunity tests were extensively used in classifying mouse encephalomyelitis virus strains.[1] Subsequent work has detracted from the reliability of such tests since interferences are

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[8] Jungeblut, C. W., personal communication.

TABLE IV.
Results of Complement-Fixation Tests.

Serum	Antigen		
	MM	GDVII	SK (Ret. 3)
McG rabbit	—	2+ (1:6)	2+ (1:24)
Lansing rabbit	—	2+ (1:6)	3+ (1:6)
GDVII rabbit	4+ (1:24)	4+ (1:12) 1+ (1:24)	3+ (1:24)
SK rabbit	—	1+ (1:6)	4+ (1:12)
MM rabbit	4+ (1:48)	4+ (1:48)	4+ (1:48)
Normal rabbit	—	—	—

The figures in parentheses indicate dilution of antigen. Sera all prepared by hyperimmunization of rabbits.

known to occur in experimental poliomyelitis.[9] We have not devised a wholly satisfactory means of separating cross immunity from interference in the case of these viruses. The chief difficulty is that if the interval between injections be greatly prolonged the test animals are usually spontaneously immune because of age.

Some of the reactions[10] are clearly due to interference while others may represent cross immunity. In those summarized in Tables II and III the likelihood of interference has been minimized in the first case by demonstrating different responses to 2 simultaneously injected challenging viruses and in the second case by using as the initial virus the MM strain which does not persist long in the hamster. In this experiment representative paralyzed and non-paralyzed animals were sacrificed and found free of transmissible infection before the challenging virus was injected.

The results of these experiments support the neutralization tests in indicating a relationship between MM and Lansing viruses but no relationship between MM and mouse encephalomyelitis viruses.

Complement-Fixation Tests. The viruses have also been compared by complement-fixing tests. The antigens were prepared from recently paralyzed hamster brains by the method of Casals and Palacios.[11] The results are shown in Table IV and are difficult to correlate with the neutralizing and cross immunity tests in that they show no reaction between MM antigen and Lansing hyperimmune rabbit serum although the latter does react with both GDVII mouse encephalomyelitis and SK viruses. On the other hand GDVII rabbit serum reacts well with MM antigen. We have been unable to prepare satisfactory Lansing antigen, probably because of the low titer of virus in paralyzed hamsters.

Discussion. A comprehensive discussion seems unwarranted at this time because the basic problem should be decided by a review of all of the applicable evidence. The results described give substantial support to the view that MM virus is a strain of poliomyelitis. If this is true it is the first strain to be adapted to rodents by hamster passage, an adaptation we have failed to repeat in several later attempts with other strains. The results also support Jungeblut's conception of a group of poliomyelitis viruses including some strains of mouse encephalomyelitis. We believe the results of the cross immunity tests to be indecisive in view of the possibility of interference but the neutralization tests yield evidence which seems trustworthy. This is particularly true because the rabbit sera used have shown a high degree of specificity. There is no evidence of broadened reactivity such as is true of anti-influenza hyperimmune rabbit sera.[12] Sera from rabbits immunized by injecting GDVII virus have been found to neutralize both MM and SK viruses but the reverse reactions do not occur.[13]

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[9] Dalldorf, G., Douglass, M., and Robinson, H. E., *Science*, 1937, **85**, 184; Dalldorf, G., and Douglass, M., Proc. Soc. Exp. Biol. and Med., 1938, **39**, 294; Jungeblut, C. W., *J. Exp. Med.*, 1945, **81**, 275.

[10] Dalldorf, G., and Whitney, E., *Science*, 1943, **98**, 477.

[11] Casals, J., and Palacios, R., *J. Exp. Med.*, 1941, **74**, 409.

Theiler[1] reported the absence of serologic relationship between mouse encephalomyelitis and Lansing poliomyelitis but relative resistance of mice paralyzed by mouse encephalomyelitis to Lansing virus. He considered the resistance non-specific because it seemed to depend on the actual presence of paralysis. We have confirmed these results. Mice seem less suitable than hamsters for experiments of the kind.

The usefulness of MM virus as a laboratory tool deserves mention. The disease in intraperitoneally injected hamsters is remarkably like human poliomyelitis. Most of the animals survive and the majority of the survivors completely recover the use of their extremities. Antibodies develop rapidly. The virus is stable in glycerin for at least 17 months. Finally MM virus seems of especial interest by being intermediate between Lansing poliomyelitis and mouse encephalomyelitis and therefore especially adapted to taxonomic studies.

Conclusions. Neutralization and cross immunity tests indicate a relationship between MM and Lansing poliomyelitis but no relationship between MM and TO (Theiler original or low virulence strains) mouse encephalomyelitis. Complement-fixation tests relate MM virus and the GDVII strain of Theiler's virus. The results support the view that a group of poliomyelitis viruses exists which may be related by serologic tests.

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[12] Francis, T., Jr., and Magill, T. P., *Brit. J. Exp. Path.*, 1938, **19**, 284.

[13] Jungeblut, C. W., *Am. J. Pub. Health*, 1944, **34**, 259.

15015

Further Investigations on the Recovery of Inhibited Conditioned Reactions.*

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It has been shown in several preceding investigations[1] that conditioned reactions (c.r.) which had been inhibited in rats by lack of reinforcement can be re-established without further training process by subjecting the animals to either insulin coma or electrically induced convulsions. In order to gain insight into the neurophysiological processes underlying this effect it was thought desirable to extend the investigations to animals in which more than one c.r. had been established.

Method. Successful experiments were carried out on 10 rats. In some of these 2 to 3 c.r.'s were established in succession. However, no new c.r. was formed until the preceding c.r. had been inhibited by lack of reinforcement. A doorbell, a sound of 250 vibrations per second, and a light served as conditioned stimuli (c.s.). A shock applied to the grid on which the rat was standing was the unconditioned stimulus and the c.r. was an escape reaction as described in the previous papers.

In the second group of animals an attempt was made to maintain one conditioned reaction at about 100% and to inhibit another conditioned reaction without influencing the first. Such animals were used in order to decide the question as to whether the action of insulin coma and electrically induced convulsions is

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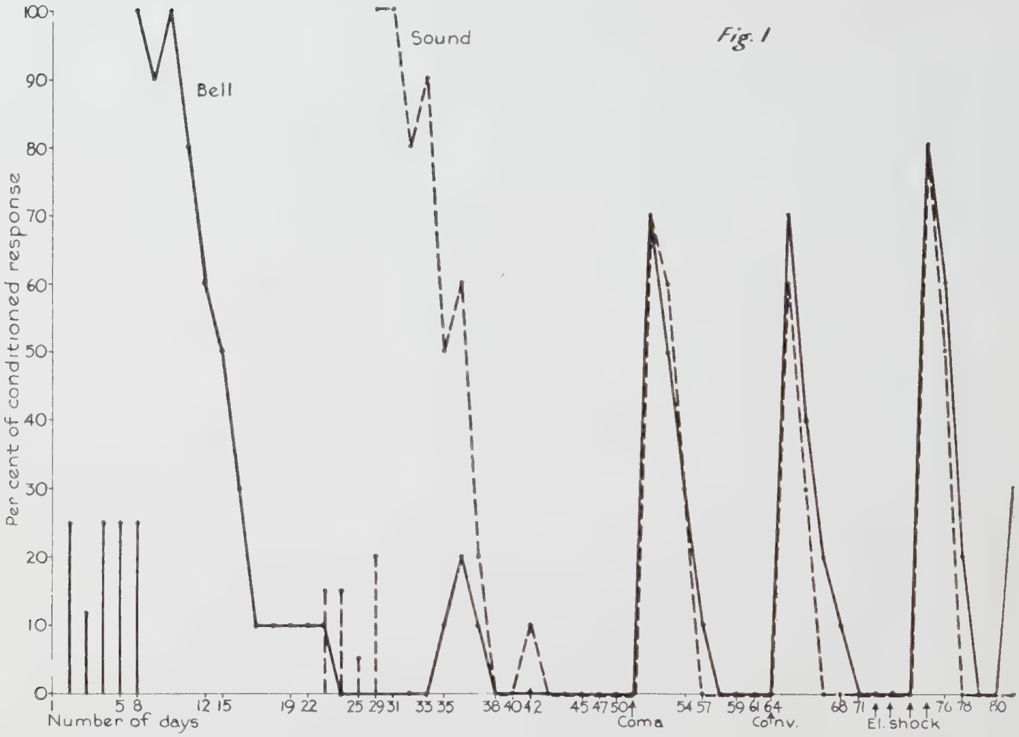
* Aided by a grant from the Josiah Macy, Jr., Foundation.

[1] Gellhorn, E., Kessler, M., and Minatoya, H., PROC. SOC. EXP. BIOL. AND MED., 1942, **50**, 260; Gellhorn, E., and Minatoya, H., *J. Neurophysiol.*, 1943, **6**, 161; Kessler, M., and Gellhorn, E., *Am. J. Psychiat.*, 1943, **99**, 687; Gellhorn, E., *J. Lancet*, 1943, **63**, 307.

Fig. 1



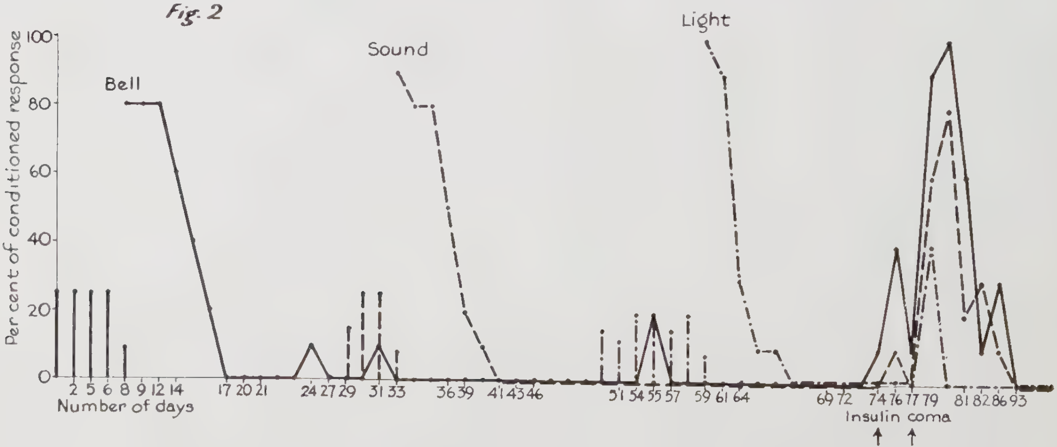
Effect of insulin coma, insulin convulsions and electroshock on the recovery of inhibited conditioned reactions of the rat.

——————— bell is conditioned stimulus.

— — — — — "250" sound is conditioned stimulus.

The heavy and interrupted vertical lines indicate the number of reenforced conditioned stimuli which established conditioning in response to bell and "250" sound respectively.

Fig. 2



The effect of insulin coma on the recovery of 3 inhibited conditioned reactions. Note that the conditioned reaction to the light recovers much less than that in response to the acoustic stimuli.

restricted to inhibited c.r.'s, or exerts a depressant effect on non-inhibited c.r.'s.

Results. I. *The Effect of Insulin Hypoglycemia and Electroshock on Several Inhibited Conditioned Reactions.* Fig. 1 to 4 are chosen as representative experiments in which

TABLE I.
Effect of Insulin Convulsions on the Restitution of Two Successively Inhibited Conditioned Reactions.

	Bell	Light
1. No. of reenforced conditioned stimuli	61	130
2. Degree of conditioning (%)	100	80
3. Duration of inhibition period (days)	49	9
4. Recovery of c. r. through insulin convulsions (%)	80	40

the effect of insulin hypoglycemia and electroshock was studied on the restitution of several inhibited c.r.'s. All figures confirm our earlier investigations in that electroshock and insulin hypoglycemia in the form of coma or convulsions restore previously inhibited c.r.'s but they demonstrate also that this effect may be shown simultaneously on several c.r.'s which had been established and inhibited in a definite sequence.

Fig. 1 shows the effect of insulin coma, hypoglycemic convulsions and electroshock on the restoration of previously inhibited c.r.'s in which the bell and the "250" sound were used as c.s. In this experiment one coma or one insulin convulsion was sufficient to induce a considerable recovery of the previously inhibited c.r. whereas several electroshocks given on successive days were necessary to produce a similar effect. The degree of restitution is similar for both types of c.r. studied in this experiment.

The experiment represented in Fig. 2 in which 3 c.r.'s were successively established and inhibited reveals after insulin coma some peculiar quantitative differences in the re-establishment of the previously inhibited c.r. After the coma the c.r. to light, to the "250" sound, and to the bell are temporarily re-established but contrary to what one might expect, the reaction to the bell is restored most and that to the light least, in spite of the fact that the last reaction to be established and inhibited before the hypoglycemic coma was employed was that to the light. It is worthy of mention that the reaction to the bell could be re-established to 100% in spite of the fact that it had been completely inhibited for two months.

Table I and Fig. 3 and 4 give further examples of the recovery of 2 inhibited c.r,'s after insulin hypoglycemia and electroshock. In the experiments of Table I and Fig. 3 the c.r. to the bell was first established and inhibited and the same processes were induced with the light as c.s. In another experiment (Fig. 4) the same procedure was employed in the reverse sequence. The experiments show clearly that the results are independent of the temporal sequence. The restitution, after hypoglycemic coma, of the c.r. to the bell as c.s. is always greater than that seen in response to the light.

Fig. 4 shows also that during the training period 220 combinations of light and shock failed to establish a c.r. The conditioning test performed at the end of this period gave a positive response of only 10%. Hereafter, a conditioned reaction to the bell was established with 80 combinations of bell plus shock. Although the light was not employed as a c.s. during this period a positive reaction to the light was seen in 50% of the responses at the end of the training period involving the bell as c.s. Apparently processes of irradiation (Anrep[2]) are not entirely confined to stimuli belonging to the same modality.

From these and other experiments not illustrated in this paper, we know that it is far more difficult to establish a c.r. to a light than to a sound in the rat. Moreover, the c.r. to the light is more easily inhibited by lack of reinforcement than the c.r. established in response to a sound. Taking these data into account, it seems to follow:

1. That the degree of temporary recovery of inhibited conditioned reactions is within fairly wide limits independent of the temporal sequence in which the reactions had been established. 2. That the degree of recovery is related to the stability of the conditioned reaction prior to its inhibition by lack of reenforcement.

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[2] Anrep, G. V., *Proc. Roy. Soc. London, Ser. B*, 1923, **94**, 404.

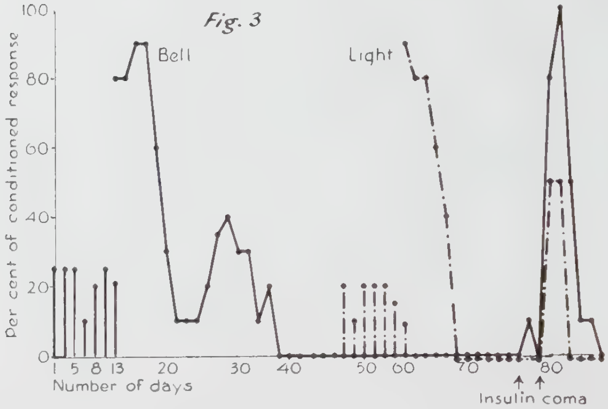


Fig. 3

The effect of insulin coma on the recovery of 2 inhibited conditioned reactions.

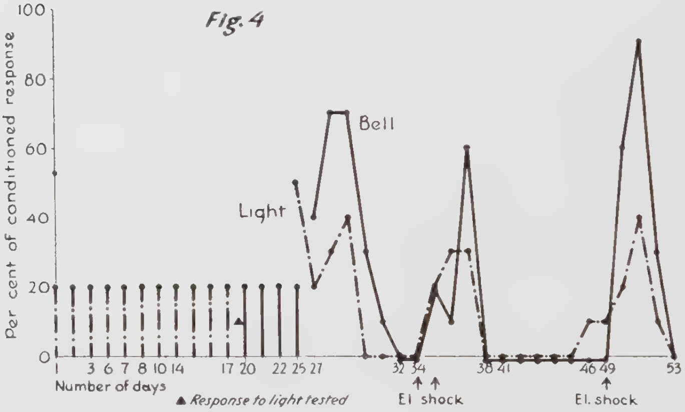
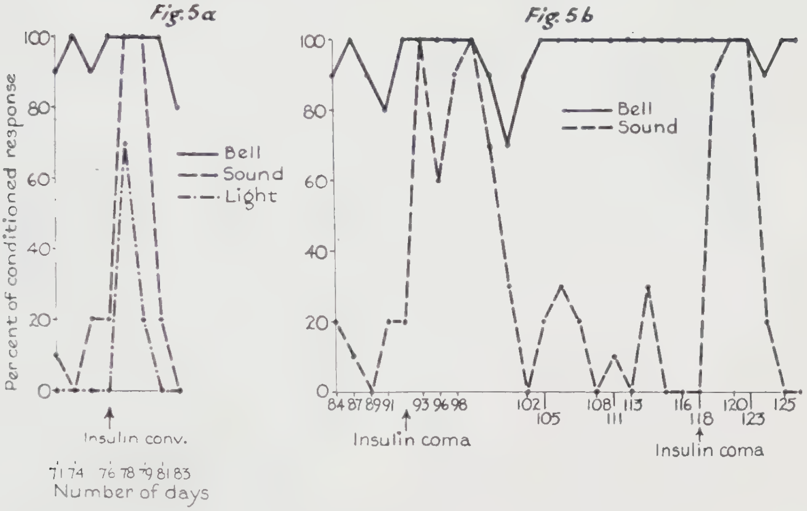


Fig. 4

The effect of electroshock on the recovery of 2 inhibited conditioned reactions.

II. *Experiments on Rats with Positive and Negative Conditioned Reactions.* In several instances experiments were performed in which we utilized the degree of stability of different c.r.'s in order to establish positive conditioned reactions and negative (inhibited) c.r.'s. In other experiments, one c.r. was more inhibited than another in order to maintain a positive c.r. of the latter but a negative c.r. of the former. The experiments of Fig. 5 may serve as an example.

In the experiment of Fig. 5A, a conditioned reaction to the bell was first established. This was followed by the establishment of a conditioned reaction to the "250" sound and finally to the light. Before the c.r. to the "250" sound was established, the c.r. to the bell had been extinguished by lack of reenforcement. When the reaction to the "250" sound was being established there was a decided state of irradiation (*cf.* Anrep) during which not only the reaction to the "250" sound but also to the bell became maximal. The animal was very alert and a 100% c.r. to the light was established in 4 days. Then the rat was tested for 3 weeks. This procedure usually leads to a complete loss of the c.r. in a few days. In this animal, however, the c.r. was retained almost completely. It was therefore decided to increase the rate of inhibition by subjecting the animal to an increased number of non-reenforced conditioned stimuli. This was done with the "250" sound only, with the result that the c.r.'s to this stimulus as well as to the light were quickly abolished, whereas the more stable reaction to the bell remained unchanged. Fig. 5A shows that at the beginning of the

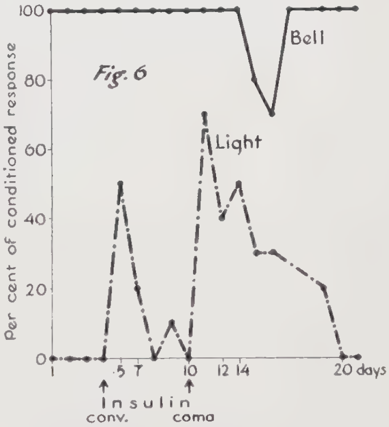


Represent sections of 2 experiments in which the conditioned reaction to the bell was maintained at 100% whereas the conditioned reaction to the light and to the "250" sound was inhibited. It is shown that under the influence of insulin coma and hypoglycemic convulsions the inhibited conditioned reactions are restored but the positive conditioned reaction to the bell remains uninfluenced.

record the reaction to the light was completely negative and that to the "250" sound varied between zero and 20%, whereas the reaction to the bell was strongly positive (90 to 100%). Insulin convulsions were induced and resulted in a temporary restitution of the c.r. for the "250" sound and for the light, while the c.r. to the bell remained uninfluenced and positive at 100%.

The story of the experiment, part of which is shown in Fig. 5B, is somewhat similar. Here again it was found that when the c.r. to the "250" sound was established, the reaction to the bell which had been inhibited, became positive again. The rat was tested for several weeks for its reaction to the bell and the "250" sound and again, contrary to the general rule, the c.r. remained positive for a long time. Eventually, however, the reaction to the "250" sound declined while that to the bell remained at a level varying between 80 and 100%. Thus the animal could be tested for the effect of insulin coma on the positive c.r. (bell) and the negative c.r. ("250" sound). As Fig. 5B shows, the hypoglycemic coma led to a temporary recovery of the inhibited reaction to the "250" sound without influencing the positive c.r. to the bell.

In Fig. 6 a similar experiment is presented with the modification that the positive and negative c.s. belonged to different modalities (acoustic and optical respectively). Prior to the administration of insulin the reaction to the light was zero and that to the bell 100%. Insulin coma caused the reaction to the light to recover up to 50% during the first experiment and up to 70% in the second experiment. In both cases the reaction to the bell remained at 100%. Only 5 or 6 days after the administration of insulin did the reaction to the positive c.s. decline slightly. From the time rela-



The effect of insulin coma and convulsions on the positively conditioned reaction to the bell and the negatively (inhibited) conditioned reaction to the light.

tions it appears likely that this effect is not due to insulin hypoglycemia but rather due to some irradiation of inhibition associated with the decline in the c.r. to light. The experiments show clearly that insulin coma and convulsions act specifically on inhibited c.r.'s without influencing positively conditioned reactions.

Further experiments on animals with partially conditioned reactions support this interpretation. At first the c.r.'s to the bell and the "250" sound were fully established and then inhibited. Different degrees of inhibition were obtained with the procedures mentioned previously. In the first animal we succeeded in maintaining a c.r. to the bell varying between 50 and 60% whereas the reaction to the "250" sound was inhibited, the response varying between zero and 10%. This condition persisted for 10 days prior to the administration of insulin which caused a coma. During the following 2 days the reaction to the bell rose to 100% and that to the sound to 60%. In another experiment the c.r. to the bell varied between 30 and 40% whereas that to the "250" sound was completely negative. This condition was present for seven days before insulin was administered. Hypoglycemic coma induced the temporary restoration of the reaction to the bell to 100%, and to the "250" sound up to 60%. From these experiments it follows that the degree of restitution of inhibited conditioned reactions is somewhat related to the degree of inhibition. No inhibitory effect was found in any of these experiments on the positive conditioned reactions.

Discussion. The experiments show that the degree of restoration of an inhibited c.r. depends on the stability of the c.r., which, other conditions being equal, is determined by the nature of the c.s. Thus it was found that a c.r. in response to an acoustic stimulus was restored to a higher degree than a c.r. established in response to a light. This holds true even for those cases in which prior to the inhibition both c.r.'s had been perfected to a similar degree. The duration of the period of inhibition seemed to be of little importance.

Observations on positive and negative c.r.'s showed conclusively that insulin hypoglycemia and electroshock restored the inhibited c.r.'s without diminishing the positive c.r.'s. The latter result differs from that of Rosen and Gantt[3] who studied the effect of repeated metrazol convulsions on conditioned reflexes in 4 dogs and found in 3 of their dogs a diminished differentiation and in one dog which was characterized as a labile hyperexcitable animal an improved differentiation on the days when metrazol was injected. Rosen and Gantt interpreted the work of Gellhorn, Kessler and Minatoya as being in complete agreement with their own since in both instances inhibited reactions were converted into excitatory c.r.'s. However, the new experiments presented in this paper clearly show a fundamental difference between the study of Rosen and Gantt and our own work since restoration of previously inhibited c.r.'s is not accompanied by a loss or diminution of the positive (non-inhibited) c.r.'s. In other words, there is no indication whatever that a dedifferentiation characterized by a loss of conditioning of the positive c.r. and a gain of the negative (inhibited) c.r. is caused by insulin hypoglycemia and electroshock in our animals. The dedifferentiation observed by Rosen and Gantt may be due to the fact that dogs are more sensitive to convulsions than rats and that a relatively long series of metrazol injections was employed.

Summary. The recovery of inhibited conditioned reactions occurring under the influence of insulin hypoglycemia, and electroshock which was described earlier was subjected to further experimental analysis. It was found that:

1. Several conditioned reactions which had been established and inhibited in a definite temporal sequence may recover simultaneously under the influence of hypoglycemic coma or convulsions or as the result of electrically induced convulsions. The degree of recovery is related to the degree of stability of the conditioned reaction prior to its inhibition but is within wide limits independent of the time which has elapsed since the conditioned reaction had been established.

2. The restitution of inhibited conditioned reactions is not accompanied by a change in

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[3] Rosen, V. H., and Gantt, W. H., *Arch. Neurol. Psychiat.*, 1943, **50**, 8.

the response of positively established conditioned reactions.

It is concluded that insulin coma and electroshock may act specifically on inhibited conditioned reactions without causing dedifferentiation.

I am much indebted to Miss Kathryn Seese for her help in this work.

15016

Postmortem Studies on Hypertensive Rats Chronically Intoxicated with Lead Acetate.

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Detailed descriptions of the clinical picture of chronic lead poisoning are found in almost all acceptable textbooks of general medicine. The accompanying hypertension in some cases of the disease has been the subject of many publications, but this problem is still unsolved. Exhaustive studies on the pathologic changes that may occur in chronic plumbism, especially among humans, have served to a certain extent to complicate the problem. Vigdortchik[1] states that "nephrosclerosis is not necessarily a companion of lead hypertonia; in most cases hypertonia is accompanied by no kidney modifications although work with lead undoubtedly predisposes to some kidney trouble and to nephrosclerosis in particular. Such kidney modifications in lead cases occur about 3 times as often as in non-lead cases." Belknap[2] denies the presence of hypertension among human beings chronically intoxicated with lead and in contact with the poison for a period of 5 to 20 years. Other investigators[3,4,5] claim that lead is perhaps the only vascular poison, and intoxication in man leads to hypertension not only during the acute episodes, but also in chronic plumbism. They agree that prolonged exposure to the poison results in an arteriosclerotic nephritis.

Fouts and Page[6] failed to produce hypertension in dogs chronically intoxicated with lead. They suggest further studies of this phenomenon on human beings since the evidence on which it is based is very slim. Dominguez failed to produce hypertension in rabbits with either lead acetate or lead carbonate.[7] Nevertheless, Griffith and Lindauer[8] were successful in producing a hypertonia in rats within 30 to 80 days after daily administration of 70 mg of lead by stomach tube.

The object of the experiment reported herein was to study the pathologic changes that occur in the tissues of rats which have developed hypertension following chronic lead poisoning.

Method. Thirty young and healthy albino rats were employed in this experiment. Lead acetate in doses of 90 mg (70 mg of lead) was administered daily for 50 days with omission of every 7th day. The poison was dissolved in 2 cc of water and was given by stomach tube.

Before the administration of the first dose of lead the systolic blood pressure of all rats was measured by the indirect method devised by Griffith.[9] These readings varied between 90 and 130 mm Hg. Subsequent blood pres-

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[1] Vigdortchik, N. A., *J. Indust. Hyg.*, 1935, **17**, 1.

[2] Belknap, E. L., *J. Indust. Hyg. and Toxicol.*, 1936, **18**, 380.

[3] Fishberg, A. M., *Hypertension and Nephritis*, Ed. 4, 1939, Lea and Febiger Co., Philadelphia.

[4] Zadek, E., *Z. f. Klin. Med.*, 1931, **116**, 241.

[5] Teleky, cited by Zadek.[4]

[6] Fouts, P. J., and Page, I. H., *Am. Heart J.*, 1942, **24**, 329.

[7] Dominguez, R., *Arch. Path.*, 1928, **5**, 577.

[8] Griffith, J. Q., Jr., and Lindauer, M. A., *Am. Heart J.*, 1944, **28**, 295.

[9] Griffith, J. Q., Jr., Proc. Soc. Exp. Biol. and Med., 1934, **32**, 394.

TABLE I.
Effect of Chronic Lead Poisoning on Systolic Blood Pressure of 11 Rats. (Normal systolic pressure 90 to 130 mm of Hg.)

	Day of experiment	
	45th day B.P.	60th day B.P.
Rat No. 1	186	202
2	180	194
3	150	190
4	144	210
5	170	200
6	110	176
7	130	170
8	150	174
9	140	188
10	136	170
11	110	130

sure determinations were made in each of the animals that survived the intoxication on the 45th and 60th days. All animals were anesthetized with ether during the procedure.

At the conclusion of the experiment the organs were removed and immediately fixed in Zenker-formol solution.

Results and Discussion. Ten of the 11 animals that survived 60 days developed hypertension (Table I).

At autopsy it was our impression that the hearts were enlarged, apparently as the result of minor degrees of both hypertrophy and dilatation. However, they were not weighed. This finding was equivocal in the rat which failed to develop hypertension. The kidneys also appeared larger than normal and their external surfaces were mottled and pale, but the normal markings were distinct on the cut surfaces. Two rats had a unilateral hydronephrosis, the cause of which was not determined. Gross pathologic changes were not observed in any of the other viscera.

The heart, kidney, and liver were studied microscopically in all cases, the aorta in 10, the renal arteries and veins in 4, the adrenal gland in 7, and the lungs in 5. The hearts of the hypertensive rats showed histologic changes consistent with a slight degree of hypertrophy. Except for this, significant histologic findings were limited to the kidneys and liver.

The most striking finding was the presence of solid and rounded, or irregular and granular, basophilic deposits in the epithelium of the renal tubules. These deposits, which were blue-black in hematoxylin and eosin preparations, usually occurred in the cytoplasm of the cells lining the convoluted tubules in the region of the junction of the cortex with the pyramid, frequently surrounding or partially obscuring the nuclei. A few were also present in the capillary endothelial cells. The bodies were stained blue-gray with fresh, unripened hematoxylin and were deeply basophilic in methylene blue preparations. These tests are considered indicative of the presence of lead by Mallory and Parker[10] although they are not entirely specific. These authors recommend fixation in 95% or absolute alcohol whereas our material was fixed in Zenker-formol. Precipitation with H_2S was not attempted. The inclusions were not stained by von Kossa's method for calcium salts. It seems likely that these inclusions represent lead or lead salt deposits. Rauh[11] has described lead deposits (demonstrated by H_2S precipitation) in the epithelium of the loops of Henle in the kidneys of guinea pigs given lead orally. He observed similar deposits in the medial coats of blood vessels and in capillary endothelium only after subcutaneous administration.

Acidophilic intranuclear inclusions, such as described by Blackman[12] as occurring in the renal tubular epithelial cells and hepatic cells, were noted in the kidneys of 3 animals of this series. These inclusions were all small and only a few were found after prolonged search.

Similarly, morphologic changes indicative of destruction or regeneration of the tubular lining cells, as described also by Blackman, were very scanty. In the kidney of only one animal were irregular, elongated or hypertrophied cells and mitotic figures at all conspicuous. Blackman's studies were made on poisoned children, but similar observations in rats have been recorded by Finner and Calvery.[13] Fairhall and Miller,[14] however, have demonstrated that such cellular alterations in the kidneys of rats disappear rapidly after return to a normal diet. Our rats had received no lead in the 10 days prior to sacrifice.

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[10] Mallory, F. B., and Parker, F., Jr., *Am. J. Path.*, 1939, **15**, 517.

[11] Rauh, F., *Arch. f. Exp. Path u. Pharmakol.*, 1934, **174**, 352.

[12] Blackman, S. S., Jr., *Bull. Johns Hopkins Hosp.*, 1936, **58**, 384.

[13] Finner, L. L., and Calvery, H. O., *Arch. Path.*,

Basophilic deposits similar to those in the kidney were also seen in the liver, more commonly, however, in Kupffer cells than in hepatic cells. No intranuclear inclusions were seen in this organ.

These basophilic cytoplasmic bodies were observed in the kidneys and liver of the non-hypertensive rat as well as in the other animals.

The arterioles and arteries of the kidneys, the aorta, and the blood vessels of the other organs studied showed no histologic abnormalities.

Summary. An experiment is reported which confirms the successful production of hypertension in rats by the administration of lead for prolonged periods.

Post-mortem studies on 10 hypertensive rats and one similarly treated rat, which failed to develop hypertension, revealed no significant blood vessel alterations.

The most striking and consistent finding was the presence of basophilic cytoplasmic inclusions, which may represent lead or lead salts, most commonly in the tubular epithelial cells of the kidney and the Kupffer cells of the liver.

Renal changes similar to those described by others in chronic lead intoxication in both humans and experimental animals were also observed but were irregular and inconspicuous features.

1939, **27**, 433.

[14] Fairhall, L. T., and Miller, J. W., *Pub. Health Rep.*, 1941, **56**, 1641.

15017

Oxidation-Reduction Potential Requirements of *Cl. welchii* and Other Clostridia.*

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Previous work[1,2] has shown that the limiting oxidation-reduction potential for growth of *Bacteroides vulgatus* and of *Cl. sporogenes* is at Eh + 0.150 volt at pH 6.6; at potentials more positive than this limiting value the organisms do not grow, while at more negative potentials they grow consistently on a suitable nutrient medium at this pH. This study is concerned primarily with the effect of pH on the limiting potentials for growth of *Cl. welchii, Cl. sporogenes,* and *Cl. histolyticum.*

Experimental Methods. In general the procedure is to maintain constant the Eh and pH of an inoculated medium for 10 to 30 hours, and to distinguish those Eh and pH values at which growth occurs, from those at which growth does not occur. The apparatus and procedure have been previously described.[1,2] The electrode culture vessels consist of 100 ml wide mouth bottles, each of which contains 75 ml of medium, and is fitted with a rubber stopper which supports gas inlet and outlet tubes, glass electrode, platinum electrodes, and salt bridge connection to a calomel cell. After autoclaving, the pH of the medium is adjusted to the desired level by addition of HCl or NaOH, 0.2 ml of a brain broth culture inoculum is added, glass electrode potentials are observed every half hour, platinum electrode potentials every 10 minutes, and the time of the first appearance of growth in any vessel is carefully noted. Previous reports[1,2] should be consulted for details of sterilizing and inserting glass electrodes, establishing sterile salt bridge connections, stirring, use of poising agents, criteria for growth, and tests for contamination.

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* Aided by a grant from the Dr. Wallace C. and Clara A. Abbott Memorial Fund of the University of Chicago. Acknowledgment is also due for a grant from the Ella Sachs Plotz Foundation.

[1] Vennesland, B., and Hanke, M. E., *J. Bact.*, 1940, **39**, 139.

[2] Hanke, M. E., and Katz, Y. J., *Arch. Biochem.*, 1943, **2**, 183.

Cultures of all 3 organisms were obtained from the Department of Bacteriology, University of Chicago. In addition, a culture of *Cl. welchii* was obtained from Dr. Hac,[3] of the Department of Obstetrics and Gynecology. The limiting potentials of these 2 strains of *Cl. welchii* were found to be the same. Best growth was obtained from an 18-hour subculture in the case of *Cl. welchii,* and 24 hours in the cases of *sporogenes* and *histolyticum.* In addition to the peptone medium previously used (10 g glucose, 5 g NaCl, M/15 phosphate, 5 g peptone, 3 g meat extract, in 1 liter), a gelatin yeast medium was also employed (10 g glucose, 5 g NaCl, 0.1 g $MgSO_4$, M/15 phosphate, 10 g gelatin, 5 g yeast extract, in 1 liter). The limiting potentials found with these two media were identical.

In any experiment, at least 3 (sometimes as many as 8) vessels were inoculated from the same culture, potentials were controlled, and observations were made on growth or lack of growth, simultaneously in all 3 (or more) vessels. In one of these vessels, strict anaerobic conditions were maintained, so that growth occurred in a minimum time, which was about 5 hours in the case of *Cl. welchii,* 6 to 10 hours in the case of *sporogenes,* and 8 to 12 hours in the case of *histolyticum.* In the other vessels the Eh values were controlled and maintained at 2 or more different levels, so that, in one vessel at a given pH growth would occur, and in another vessel at the same pH at a more positive Eh growth would not occur. In other pairs of vessels, the Eh value was kept the same in both, while the pH was varied so that growth occurred in one, but not in the other of a pair. In some experiments, after it was established that growth did not occur under a given set of conditions, the Eh value was adjusted to a more negative (a more favorable) value, and then growth was observed. In this way the limiting pH and Eh values for growth could be established with confidence. In the controlled vessels the time required for growth was usually greater than in the strictly anaerobic vessels, although experiments were usually not carried on for times longer than twice the time required for growth in the anaerobic controls. It should perhaps be emphasized that the success of these experiments depends upon constant and continuous potentiometric observation and control of pH and Eh for 12 to 30 hours. This was accomplished by having a crew of 3 or 4 people working 3- or 4-hour shifts.

In most experiments the Eh values were controlled by bubbling through the medium controlled proportions of air and nitrogen, as described by Vennesland and Hanke;[1] in a few cases the electrolytic method of Hanke and Katz[2] was used. The former is more satisfactory for determination of the limiting potential chiefly because it avoids the changes in pH which follow the electrolytic formation of acid or base. Also the time required for growth at a given favorable Eh and pH is greater, when electrolytic control is used. This may be due not only to the unfavorable pH effects of electrolysis; but the local liberation of oxidizing agents at the surface of the electrode, may exert some inhibitory action on the microörganisms. When suitably carried out, so that prolonged and intensive electrolysis is avoided especially in the early part of the experiment when the inoculum is recently introduced, both methods show the same result. However, for precise determination of limiting Eh values for growth the air-nitrogen method of Eh control is preferred.

In calculating the results, the 12 ten-minute observations of the Eh in any 2-hour period are averaged. If the average deviation of these exceeds 8 millivolts the experiment is discarded as unsatisfactory. Usually the average deviation was 3 to 6 millivolts. These successive two-hour averages for a given vessel are then averaged to give the grand average for the entire period. In order to be considered satisfactory, the average deviation of these 2-hour values for the grand average must not have exceeded 8 millivolts.

Results. Table I gives the result of 60 observations on the growth or lack of growth

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[3] Hac, L. R., and Hubert, A. C., PROC. SOC. EXP. BIOL. AND MED., 1943, **53**, 58; *idem.*, 1943, **53**, 61.

TABLE I.
Observations on Growth or Lack of Growth in Cultures of *Cl. welchii* at Controlled Eh and pH Values.

	Eh in millivolts			Eh in millivolts			Eh in millivolts	
pH	Growth	No growth	pH	Growth	No growth	pH	Growth	No growth
5.78		111	6.45		166	6.86		150
5.92		155	6.45		166	6.88	114	
5.93		153	6.47	146		6.92		128
5.98		117	6.47	145		6.95	91	
5.98	92		6.49		181	6.95		107
6.03	106		6.50	141		6.96		129
6.06		114	6.55	134		6.98	84	
6.07	98		6.55		147	6.99	91	
6.07	111		6.55	141		7.06		87
6.18	131		6.57	149		7.07	67	
6.20		144	6.58		145	7.10		150
6.30	143		6.58		150	7.20		116
6.32		157	6.59		164	7.21	79	
6.35	157		6.60		191	7.26		103
6.36	146		6.60		167	7.32		57
6.38		163	6.60		166	7.35	40	
6.40	143		6.67		148	7.42		74
6.40	156		6.71	129		7.74		36
6.42		172	6.73		131	7.74		31
6.44	162		6.75		123	7.88	—12	

TABLE II.
Observations on Growth or Lack of Growth of *Cl. sporogenes* at Controlled Eh and pH Values.

	Eh in millivolts			Eh in millivolts			Eh in millivolts	
pH	Growth	No growth	pH	Growth	No growth	pH	Growth	No growth
6.08		97	6.30	136*		6.60	136*	
6.08		106	6.40	144		7.08		115
6.13	118†		6.45		152	7.09	114	
6.14		147	6.50		163	7.02	124†	
6.15	121		6.60	116		6.97		140†
6.19		145†	6.60	36		6.97	117†	
6.27	133†		6.60		155*			

* These data are taken from Hanke and Katz.[2]
† These data are taken from Katz.[4]

in cultures of *Cl welchii* at a variety of Eh levels between pH 5.8 and 7.9. Tables II and III give similar data for *Cl. sporogenes* and *Cl. histolyticum*. In Table IV these data are summarized to give the most positive Eh value at which growth will just occur (the limiting Eh value for growth) at a few rounded pH values.

From the data, the following conclusions may be drawn. (1) There is a marked difference between the oxidation-reduction potential requirements of the three organisms; at pH 6.4 *Cl. welchii* will grow at a slightly more positive Eh level (+ 160 mv) than *Cl. sporogenes* (145 to 150 mv), while *Cl. histolyticum* requires much more reducing conditions (85 to 90 mv). (2) pH has a marked effect on the limiting Eh value for growth. In the case of *Cl. welchii,* the maximum limiting Eh value of 160 mv is reached at 6.4 and falls off rapidly at lower or higher pH value. For *Cl. sporogenes* the maximum limiting Eh value is at pH 6.4 to 6.6, and falls off more slowly with increasing pH than that of *Cl. welchii,* so that at pH 7.0, *Cl. sporogenes* will grow at

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[4] Katz, Y. J., Ph.D. Dissertation in Biochemistry, University of Chicago, 1941.

TABLE III.
Observations on Growth or Lack of Growth of *Cl. histolyticum* at Controlled Eh and pH Values.

	Eh in millivolts			Eh in millivolts			Eh in millivolts	
pH	Growth	No growth	pH	Growth	No growth	pH	Growth	No growth
6.38	63		6.54		119	6.73		139
6.42		105	6.58	94		6.98	60	
6.43		94	6.58	76		7.00		76
6.43		96	6.61		95	7.00		76
6.45	85		6.63	85		7.00		118
6.52		84	6.68	72		7.05	60	

TABLE IV.
Summary of Limiting Eh Values for Growth of three Clostridia at a Few Rounded pH Values.

Cl. welchii		*Cl. sporogenes*		*Cl. histolyticum*	
pH	Eh	pH	Eh	pH	Eh
6.0	106	6.0	<100		
6.2	131	6.2	130		
6.4	160	6.4	144	6.4	85
6.6	150	6.6	136-152	6.6	90
6.8	114				
7.0	90	7.0	114	7.0	60-76
7.2	80				
7.4	35-70				
7.8	0-30				

a more positive Eh (114 mv) than will *Cl. welchii* (90 mv). *Cl. histolyticum* also requires a more negative Eh value at pH 7.0 than at pH 6.4 or 6.6.

Certain minor inconsistencies in the data do not detract from the above conclusions. They do indicate the limit of accuracy of this type of experimentation. In Table I at pH 6.57 growth occurred at Eh 149 millivolts, but not at pH 6.58 Eh 145; at pH 6.71 growth occurred at Eh 129, but not at pH 6.75 Eh 123. In Table III growth fails at pH 6.52 Eh 84 mv, while it does occur at pH 6.58 Eh 94 mv. No inconsistencies greater than 10 millivolts have ever been observed, and this, it is felt, represents the maximum error of the conclusions.

Similar experiments were carried out with *Cl. tetani* but satisfactory quantitative conclusions could not be drawn because of the long time required for growth. In the anaerobic controls growth occurred at Eh-50 to 150 millivolts, in 8 to 12 hours, but when small amounts of air were admitted to maintain the Eh value at a more positive level, the time required for growth was increased to 40 or 50 hours. In 4 successful experiments it was observed that at pH 6.5, *Cl. tetani* will grow at Eh 85 millivolts but not at Eh 120; at pH 7.2 it will grow at Eh 55 millivolts but not at Eh 120. From this it seems that *Cl. tetani* requires more reducing conditions than *Cl. welchii* or *Cl. sporogenes,* and is more like *Cl. histolyticum* in its Eh requirements.

Summary. The oxidation-reduction potential requirements of three clostridia have been determined at a variety of pH values. The observations reveal striking and characteristic differences between the three kinds of organisms and a marked effect of pH on the limiting Eh for growth.

Acknowledgment is hereby made to the following students whose collaboration made this study possible: Richard Boyagian, Charles Gabelman, Eugene Goldwasser, Ruth Hanke, Alan Robertson, and Louis Thomas.

15018

Effect of Radiation on Malaria. An Experimental Study in the Chick and Duck.*

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Skinner and Carson[1] reported several cases of malarial infection in man in which roentgen irradiation was applied to the skin over the spleen. This method of therapy in malaria has not been widely accepted, although these investigators were favorably impressed with their results. Reinhard[2] considered the effect of radiation to be favorable in clinical cases of malaria. Twenty cases of splenomegaly the result of either chronic recurrent or repeated attacks of malaria were treated by roentgen irradiation between 1931 and 1939 by one of us (H. R., Jr.). The splenic tumor was reduced in size in each of the 16 cases that completed the series of treatments. The spleen either decreased in size or returned to normal in all of these cases. The general health of each patient improved following this therapy.

Experiments were performed to determine the effect of radiation on malaria during the time other studies were being made on *P. lophuræ* infection in ducks and chicks.[3] The results of these experiments are reported in this paper.

Methods and Materials. The strain of plasmodium used was *P. lophuræ*. These parasites were serially transferred in young chicks for approximately 6 months preceding the time in which they were used in this experiment to inoculate young chicks. The chicks were 3 to 10 days of age when inoculated. *P. lophuræ* obtained from ducks were used to infect young white Pekin ducklings. The ducks varied in age from 2 to 6 weeks.

Blood for the inoculum was obtained directly from the heart. It was diluted with an equal volume of a 2.0% solution of sodium citrate in physiological saline. Blood from a group of chicks was pooled when a large series of birds were to be inoculated. Injections were made into a vein in the leg. The course of the infection was followed by counting the number of parasitized cells per 500 red blood cells. Blood for these counts was obtained from the toes at varying intervals during the disease. Smears were stained with a combination of Giemsa's and Wright's stains.

Citrated parasitized blood was put into petri dishes approximately 10.0 cm in diameter and varying quantities of roentgen irradiation was given over these dishes. The amount of radiation is given in each experiment. The physical factors were: half-wave, K.V.P. 100, M.A. 5, with 0.5 mm Al. inherent filtration.

In a second group of experiments the effect of roentgen irradiation on malarial parasites was studied *in vivo*. Chicks were infected with *P. lophuræ* by intravenous inoculation. The entire body was irradiated. The amount of irradiation and the number of treatments varied. These data are given in the report of each experiment and are shown in Fig. 2.

The effect of radiation from radium bromide† was studied both *in vitro* and *in vivo*. In the former experiment parasitized

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* Aided by a grant from the John and Mary R. Markle Foundation to the Department of Pathology, University of Arkansas, School of Medicine. A portion of this work was done at the University of Tennessee, Memphis. We wish to acknowledge the assistance given by Dr. B. A. Rhinehart, Radiologist, Little Rock, Arkansas, and W. L. Cloyd and James Hall, students at the University of Tennessee. Research paper 544, Journal Series, University of Arkansas.

[1] Skinner, Bruce, and Carson, H. W., *Brit. Med. J.*, 1911, **1**, 431.

[2] Reinhard, P., *Med. Klin.*, 1918, **14**, 619.

[3] Rigdon, R. H., *Am. J. Trop. Med.*, 1944, **24**, 371.

† The Canadian Radium and Uranium Corporation of New York supplied the radium bromide. We wish to acknowledge our appreciation for this assistance.

blood from chicks was diluted with an equal volume of a 2.0% solution of sodium citrate in physiological saline and pooled. Six cubic centimeters of this blood was put into 2 test tubes and centrifuged. Two cubic centimeters of the supernatant was removed from each tube and discarded. Two cubic centimeters of radium bromide in distilled water was added to one of the tubes containing the parasitized blood and an equal volume of distilled water was added to the second tube of parasitized blood. These two tubes were kept at room temperature for 1½ hours. They were shaken at intervals. 0.5 cubic centimeter of these suspensions of parasitized cells were given intravenously to 20 chicks 3 days of age. The above procedure was repeated using parasitized blood cells from ducks. 0.5 cubic centimeters of this irradiated duck blood was given intravenously to 13 ducks 7 weeks of age. The parasitemia was followed with blood smears in these chicks and ducks.

To study the effect of radium bromide on *P. lophuræ in vivo* 10 ducks 14 days of age were given 0.5 cc of parasitized blood intravenously. Five of these ducks were given 1.0 cc of radium bromide intravenously 24 hours following the injection of the parasites. A second injection of 1.0 cc of radium bromide was given 30 hours and a third injection of 1.0 cc of radium bromide was given 48 hours following the injection of parasites. Five ducks in this group were carried as the controls. The parasitemia was followed by blood smears in these 10 ducks.

The radium† used in these experiments was in the form of a bromide salt dissolved in distilled water. 0.5 of a milligram of the element radium was dissolved in 30.0 cc of distilled water. The amount of this dilution given to the different birds and also mixed with parasitized red cells is given in the different experiments.

Experimental. Effect of Roentgen Irradiation on P. lophuræ in vitro. A series of experimental observations were made in which different amounts of irradiation were given over petri dishes containing parasitized blood. This blood then was injected into chicks. The results of these different experiments are shown in Fig. 1. The radiation was unfiltered. The smallest amount of irradiation was used in the experiment in which the results are shown in Fig. 1A. In this experiment, the 50, 100, and 500 R were given at the rate of 137 R/min. In experiment B 1000 R and 1500 R of irradiation were given at the rate of 126 R/min. In experiment C, 1000 R and 2000 R were given at the rate of 34.4 R/min. In experiment D, 15,754 roentgen were given over the petri dish at a rate of 787.7 R/min. The results of these experiments show that the degree of parasitemia decreases with an increase in the amount of irradiation applied to the parasitized blood. Apparently there is no reproduction of the parasites when they are treated with 15,754 roentgens preceding the time of inoculation into young chicks (Fig. 1D).

Effect of Roentgen Irradiation on the Parasitemia Produced by P. lophuræ in Chicks. The results obtained in the preceding experiments indicate that roentgen irradiation prevents the normal reproduction of malarial parasites. The effect of roentgen irradiation on *P. lophuræ* was studied *in vivo* by irradiating the entire body of chicks infected with this strain of malaria. The amount and frequency of the radiation varied in the different experiments and was given as follows: Rate 49.5 R/min, 100 K.V.P., 5 M.A., distance 10 inches. A 2 mm al. filter was used in the experiments shown in Fig. 2, A, C, and D. The rate was 137 R/min and no filter was used in the experiment shown in Fig. 2B. The frequency of irradiation and the interval following inoculation is given for each of the four experiments in Fig. 2. The results of the effect of the radiation on the parasitemia are also shown in Fig. 2. The parasitemia is less in the irradiated chicks than in the controls in each of these 4 experiments. The greatest reduction in the number of parasites occurred in the chicks given the larger quantities of irradiation. These larger amounts of irradiation frequently were fatal to uninfected chicks.

Effect of Radium Bromide on P. lophuræ.‡

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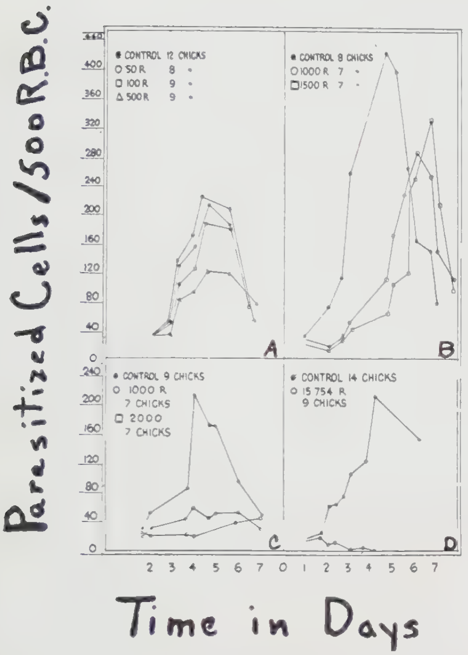
‡ Dr. Jay McLean, Columbus, Ohio, suggested to one of us (R.H.R.) the study of the effect of a radium salt on malaria.



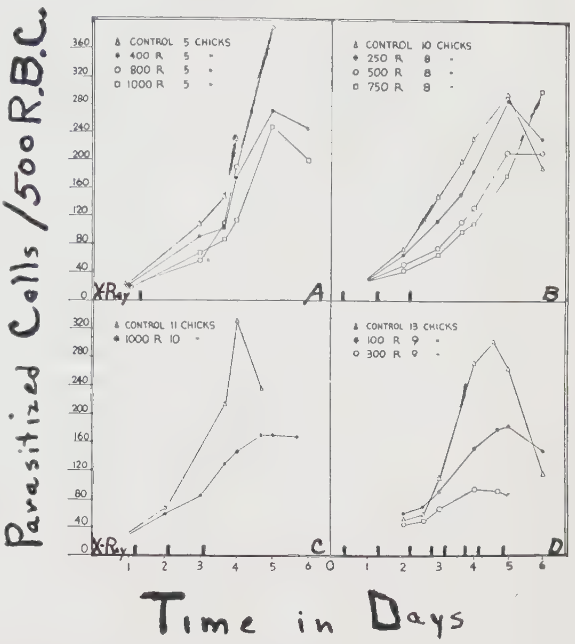


FIG. 1 (Left).

The results of the effect of roentgen rays on the malarial parasites *in vitro,* one shown in each of the 4 graphs. The number of roentgens and the number of chicks used in each experiment are shown above.

FIG. 2 (Right).

The results of the effect of roentgen rays on the malarial parasites *in vivo* are shown in each of the 4 graphs. The number of roentgens and the number of chicks used in each experiment are shown above. The frequency of the treatments and the intervals when given are indicated following the word X-ray.

There was no variation in the degree of parasitemia in the chicks and ducks injected with parasitized blood to which radium bromide was added when compared with the controls. Furthermore, the ducks with malarial infections, and given radium bromide intravenously did not show any variation in the degree of parasitemia from the control birds.

Discussion. The variation in the development of parasitemia in chicks inoculated with irradiated blood when compared with the parasitemia in control chicks indicate that the roentgen ray does injure *P. lophuræ.* Only a few parasites appear to reproduce in the blood stream of the chick when the blood is treated with 2000 roentgens preceding inoculation. Apparently all the parasites are destroyed when 15,754 roentgens are used for treating the parasitized blood. The rapid decrease in the total number of parasites in the blood stream of chicks as shown in Fig. 1D, would indicate that the parasites were dead when injected. The number of chicks used in a single experiment is small; this method for determining the degree of parasitemia is not very accurate, however, when all the data in Fig. 1 are considered they show the injurious effect of this type of irradiation on *P. lophuræ* in the chick.

The results of the *in vivo* study of roentgen rays on *P. lophuræ* in the chick as shown in Fig. 2 also indicate that this irradiation injures *P. lophuræ.* Many of the chicks died in these experiments when large amounts of irradiation were given either in multiple or single doses. The large quantity of irradiation necessary to obtain evidence of injury to *P. lophuræ* and its lethal effect on normal chicks would indicate that this form of therapy in malaria is impracticable. It would be interesting, however, to know if there is any variation in the susceptibility of different strains of plasmodia to irradiation. Furthermore, it would be interesting to know if the trophozoites varies from the sporozoites in their susceptibility to irradiation.

The quantity of radium bromide used in these experiments may have been insufficient to affect *P. lophuræ*. The amount used, however, was sufficient to produce severe degeneration in the tissues of the chick and duck.[4] The variation in the type of irradiation from radium and the roentgen rays also must be recalled in considering the differences observed in their effect on *P. lophuræ*.

The clinical observation that irradiation reduces the size of the spleen and improves the general health of the patient with malaria is of interest, however, the number of cases treated is small and the method of evaluating the results is unsatisfactory. If different stages of the plasmodium or different plasmodia vary in their susceptibility to irradiation, then irradiation of the spleeen in chronic malaria might be of some value.

Summary. *P. lophuræ* are injured by roentgen irradiation *in vivo* and *in vitro* as indicated by the development of the parasitemia in the chick. The amount of irradiation necessary to produce a significant effect *in vivo* is near the lethal dose of radiation for the chick.

Radium bromide as used in these experiments has no effect on *P. lophuræ*. Severe degenerative changes occur in the birds given radium bromide.

Since this paper was completed Bennison and Coatney have published their results of the effect of radiation on *P. gallinaceum*. (*Public Health Rep.*, 1945, **60**, 127.)

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[4] Rigdon, R. H., and Fletcher, D. C., to be published.

15019 P

Effect of Glycine on Peripheral Blood Flow.

RICHARD GUBNER AND JOSEPH DIPALMA. (Introduced by William Dock.)

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Peripheral blood flow is an important mechanism for the regulation of heat loss from the body. Under conditions of increased heat production as in fever[1] or hyperthyroidism[2] the blood flow to the periphery is greatly augmented. A linear correlation has been found to exist between the metabolic rate and peripheral blood flow over a wide range of metabolic levels in subjects with hyperthyroidism[2] and myxedema.[3]

In the present investigation, utilization has been made of specific dynamic action to increase heat production in the body. The increase in metabolic rate following a protein meal is due almost entirely to a few amino acids, notably, glycine, alanine, phenylalanine, and tyrosine; the increased heat production occurring chiefly in the liver.[4] A study was carried out to determine to what degree the dissipation of this excess heat might be attended by changes in the blood flow to the extremities.

Twenty grams of glycine* dissolved in 200 cc of water were administered to 15 subjects in the basal state. Observation was made of oxygen consumption, skin temperature of toes, fingers, and forehead, and oscillometric pulsation in the calf and forearm. Readings were made in the basal state and over a period of 4 hours following administration of glycine. Skin temperature was measured with the Leeds-Northrup potentiometer. Observations on the skin temperature were carried out under conditions of approximately constant environmental temperature (22°C ± 1.3°), and with the extremities exposed for a period

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[1] Bierman, W., *J. A. M. A.*, 1936, **106**, 1158.

[2] Stewart, H. J., and Evans, W. F., *Am. Heart J.*, 1940, **20**, 715.

[3] Stewart, H. J., and Evans, W. F., *Arch. Int. Med.*, 1942, **69**, 808.

[4] Wilhelmj, C. M., *Physiol. Rev.*, 1935, **15**, 202.

* A preparation of glycine supplied by Sharp and Dohme was employed.

TABLE I.
Average Changes in Oxygen Consumption and Skin Temperature Following Glycine (20 g).

		Cases studied (9)	Normal subjects (11)	Peripheral vascular disease (4)
Oxygen Consumption		+23.5%		
Skin Temp.:	Toe		+4.0°C	+2.3°C
	Finger		+2.8	+2.2
	Forehead		+1.7	+0.8

TABLE II.
Comparison of Skin Temperature Changes Following Glycine, Posterior Tibial Nerve Block, and Alcohol.

	6 cases		4 cases	
	Glycine	Nerve block	Glycine	Alcohol
Toe	+5.7°C	+4.5°C	+3.8°C	+1.2°C
Finger			+2.3	—0.2
Forehead			+2.4	+1.2

of 20 minutes before readings were made. Skin temperature was recorded before determination of oxygen consumption. Comparison was made with the effect on skin temperature of post-tibial nerve block in 6 cases, and of 2 ounces of whiskey in 4 cases. The observations on nerve block and alcohol were carried out on different days than the glycine experiment.

Oxygen Consumption. The average maximal increase in oxygen consumption above the basal level in 9 cases studied was 23.5%. There was considerable individual variation. Only 1 subject failed to exhibit a rise in oxygen consumption following ingestion of glycine. In another subject no significant change occurred when first tested, but when glycine was given again on another day the oxygen consumption increased 26%. The maximal increase in oxygen consumption was 52%.

Skin Temperature. (Table I) An increase in the temperature of the toes occurred in 11 cases with no impairment of peripheral circulation, and in 3 of 4 cases with peripheral vascular disease. Only one subject failed to exhibit a rise in temperature of the toes. In this case there was advanced bilateral peripheral vascular disease with acute right popliteal occlusion. The temperature of the fingers similarly increased in 14 of the 15 cases. Forehead temperature increased in 6 of the 9 cases tested. Excluding the 4 cases with peripheral vascular disease, the average rise in temperature of the toes was 4.0°C, at the fingers 2.8°C, and at the forehead 1.7°C. Significant increase in the skin temperature occurred within one hour after ingestion of glycine with maximal rise between the second and third hour. The initial temperature of the forehead was higher than the extremities. Following glycine a definite trend toward equilibration of temperature of the toes, fingers, and forehead occurred. Thus, in 6 cases with no peripheral vascular disease, in whom the skin temperature changes at toes, fingers, and forehead were recorded, the average initial temperature at toes, fingers, and forehead respectively was 26.4°C, 28.8°C, and 31.4°C. Following glycine the average maximal temperature at toes, fingers, and forehead respectively was 31.7°, 33.0°C, and 33.1°C. These temperatures indicate maximal vasodilatation. According to Montgomery *et al.*, "In the normal subject when vasodilatation is complete the skin temperature of the digits will rise to a level between 31° and 34°C."[5]

The effect of glycine was compared with posterior tibial nerve block in 6 cases (Table II). In these subjects the average increase in toe temperature was 5.7°C with glycine, and 4.5°C following nerve block. Comparison was made with the effect of alcohol in 4 subjects (Table II). In all these the increase in temperature in the toes was significantly greater with glycine than after alcohol (2 ounces of whiskey in basal state). The average change in temperature at the toes was +3.8°C with glycine, +1.2°C with alcohol; at the fingers +2.3°C with glycine, —0.2°C with alcohol; and at the forehead +2.4°C with glycine and +1.2°C with alcohol.

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[5] Montgomery, H., Naide, M., and Freeman, N. E., *Am. Heart J.*, 1941, **21**, 780.

Oscillometric Readings. The amplitude of oscillometric pulsation in the calf increased in 8 of the 11 subjects with no peripheral vascular disease. The average change for this group of 11 subjects was an increase in pulsation of 25%. No increase in oscillometric pulsation was observed in 3 cases with peripheral vascular disease although significant rises in skin temperature occurred in 2 of these subjects. In the forearm an increase in amplitude of pulsation following glycine was noted in 6 of these 11 subjects. The average increase in this group of 11 cases was 33%.

Summary. The effect of glycine on peripheral blood flow was studied in 15 subjects by means of skin temperature and oscillometric readings. The blood flow to the extremities was augmented in 11 normal subjects and in 3 of the 4 cases with peripheral vascular disease. The increase in peripheral blood flow is an accompaniment of increased heat production resulting from the specific dynamic action of glycine. The ingestion of glycine provides a simple means to accomplish an effective and sustained increase in peripheral blood flow.

15020

Relationship of Antibody Content of Normal and Malignant Lymphocytes.*

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The demonstration of antibodies in normal lymphocytes[1,2] raised the question of whether lymphocytes in lymphosarcoma also could contain antibody. At the present time there are no morphological or physiological characteristics which clearly differentiate normal from malignant lymphocytes. Since one important function of lymphocytes has recently been demonstrated to be the carrying of antibodies,[1,2] it was possible to compare malignant to normal lymphocytes in this regard.

The data reported in this communication establish the presence of antibody in the lymphocytes of lymphosarcoma, the relationship of the quantity of this antibody to that of normal lymphocytes, the capacity of tumor cells to obtain antibody from normal lymphocytes, and finally, the ability of tumor cells to carry antibody into a succeeding transplant.

Materials and Methods. Male mice 60 to 80 days old of the CBA strain (Strong) were used. The animals were fed Purina Fox Chow supplemented with calf meal; food and water were available at all times. The tumor used was obtained from Dr. W. U. Gardner. This tumor arose in an estrogen-treated mouse of the C_3H strain and has been transplanted for many successive generations. The transplant of the tumor takes in 100% of CBA mice. The transplanted tumor grows rapidly and kills the host in 2 to 3 weeks. This tumor was selected for the present experiments because it does not metastasize. This was of importance for the study because it was desired to compare the antibody content of tumor and

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* This investigation has been aided by grants from the Josiah Macy, Jr., Foundation and the Fluid Research Fund of Yale University School of Medicine.

† Alexander Brown Coxe Memorial Fellow.

[1] Dougherty, T. F., Chase, J. H., and White, A., PROC. SOC. EXP. BIOL. AND MED., 1944, **57**, 295; 1945, **58**, 135.

[2] Harris, T. N., Grimm, E., Mertens, E., and Ehrich, W. E., *J. Exp. Med.*, 1945, **81**, 73.

TABLE I.

Comparison of Staphylococcus Antitoxin Concentration in Blood and Extracts of Normal and Malignant Lymphocytes.

		Mg nitrogen/ml sera or extract			Inhibition titers			Mg nitrogen in inhibiting dilution		
	No. of mice	Serum	Lympho-cyte extr.	Tumor extr.	Serum	Lympho-cyte extr.	Tumor extr.	Serum	Lympho-cyte extr.	Tumor extr.
Exp. 1	2	5.83	3.85	—*	1:20	1:40	—*	0.276	0.096	—*
Immunized mice	1	6.44	2.58	—*	1:40	1:80	—*	0.162	0.034	—*
given tumor trans-	1	4.88	2.80	—*	1:80	1:40	—*	0.064	0.070	—*
plant; antigen con-	5†	—	—	—	1:40	1:80	1:80	—	—	—
tinued	3	7.06	4.64	8.49	1:20	1:160	1:80	0.35	0.03	0.10
Exp. 2	1	5.62	3.84	—*	1:160	1:160	—*	0.034	0.026	—*
Antigen adminis-	1	5.76	3.48	—*	1:80	1:80	—*	0.075	0.045	—*
tration started at	3	5.50	3.96	10.67	0	1:40	1:320	0	0.099	0.032
time of tumor	1	4.22	0.74	2.08	1:160	1:20	1:40	0.026	0.037	0.053
transplant	1	4.62	2.00	2.28	1:160	1:40	1:40	0.027	0.051	0.058
Exp. 3.	1	4.58	3.26	—*	1:80	1:80	—*	0.059	0.042	—*
Immunized mice	1	5.72	2.34	—*	1:40	1:80	—*	0.142	0.030	—*
given tumor trans-	2	6.22	3.54	8.19	1:20	1:40	1:160	0.31	0.088	0.049
plant; no further	1	6.10	2.40	3.50	1:10	1:80	1:80	0.61	0.032	0.045
antigen										

* The absence of data for tumor extracts indicates control mice with no tumor but otherwise treated the same as tumor-bearing animals.

† Nitrogen analyses not done in this experiment.

normal lymphoid cells in the same animal. The tumor has been described in detail[3] and is composed of large cells which resemble reticular cells. Tumor transplants were made by implanting small pieces of the tumor subcutaneously.

Since the mice with this tumor transplant live only 2 to 3 weeks, it was necessary to use an antigen which would produce significant antibody titers within this period. The antigen chosen was a filtrate of a 24-hour broth culture of *Staphylococcus aureus*. The toxin solution was injected subcutaneously in each mouse. Sera and extracts of normal and malignant lymphocytes were obtained from single or from groups of mice and were titrated for their antibody content. Tumor and normal lymphoid tissue, with the exception of the inguinal and axillary nodes nearest the tumor, were treated as previously described.[1] Nitrogen analyses of sera and extracts were made by the micro-Kjeldahl method.

Immediately before each titer estimation the minimum hemolytic dose of the toxin was determined. The antisera and the extracts were diluted according to the doubling dilution method, and the minimum hemolytic dose of toxin was added to each dilution. Then 0.2 ml of a 5% suspension of fresh, washed rabbit erythrocytes was added to each tube. The tubes were incubated in a water bath at 38°C for one hour and the titers were read. Readings were checked after the tubes had remained overnight in the ice-box. The titers were read as that dilution of serum in which hemolysis had been completely prevented.

Extracts of the tumor used in this study were shown to have no hemolytic activity when incubated with rabbit erythrocytes.

Experimental and Results. The several types of experiments which were conducted are the following:

Experiment 1. Antigen was administered to each animal on alternate days in increasing doses beginning with 0.1 ml and increasing to a maximum of 0.5 ml. At the end of 16 days the experimental mice each received a tumor transplant. Antigen was continued in both control and experimental animals for a further period of 15 days, at the end of which time all mice were bled from the heart and

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[3] Gardner, W. U., Dougherty, T. F., and Williams, W. L., *Cancer Research*, 1944, **4**, 73.

TABLE II.
Antibody Content of Transplanted Tumor Cells.

Mouse No.	Days after tumor transplant	Inhibition titers Serum*	Lymphocyte extract	Tumor extract†
A-2	13	0	1:20	1:40
B-1	13	0	1:10	1:40
B-2	15	0	1:80	1:160
B-3	15	0	1:20	1:160
B-4	15	0	1:40	1:40
B-5	15	0	1:20	1:80
A-3	17	0	1:40	1:80
A-4	17	0	1:80	1:160
A-5	17	0	1:80	1:160
A-6	17	0	1:80	1:80
A-7	17	0	1:160	1:320
A-8	17	0	1:40	1:160

* The lowest serum dilutions titrated were 1:10.
† Tumor transplant in A series had a titer of 1:80; in B series 1:40.

lymphoid and tumor tissue obtained for extraction.

Experiment 2. Antigen administration was started at the time of tumor transplant and continued for a 15-day period; the animals were then sacrificed.

Experiment 3. The mice in this experiment were immunized as described in Experiment 1. After 16 days of immunization each animal received a tumor transplant, and at this time antigen injection was stopped.

The data for the above 3 experiments are presented in Table I. In Experiment 1 the cells of the tumor contained antibody. Since histologically this tumor consists almost entirely of lymphocytes, the immune globulin must have been present in the malignant lymphocytes. The extract of normal lymphocytes contained more antibody per unit of nitrogen than that of tumor cells. In Experiment 2 antigen administration was begun at the time of tumor transplant. Consequently, there was equal opportunity for antibody appearance in both normal and malignant lymphocytes. Under these circumstances the data show that in one experiment with sera and tissues pooled from 3 animals the rapidly growing lymphosarcoma cells had a higher antibody content than did the normal lymphocytes. The other 2 animals in this experiment were moribund at the time they were sacrificed. It is interesting to note that these animals showed highest titers in the serum.

Experiment 3 illustrates the ability of tumor cells to obtain antibody from normal lymphocytes. Inasmuch as antigen was not administered following tumor transplantation into the immunized mice, the tumor cells could have obtained their antibody only as a consequence of the presence in the animal of previously existing immune globulin.

The data in Table II demonstrate that antibody-containing tumor cells will transfer immune globulin into a succeeding transplant. The mice in the A series received a tumor having a titer of 1-80; in the B series the tumor transplant had a titer of 1-40. Therefore, extracts of tumor transplants showed titers higher than those present in the tumor at the time of its transfer. The new lymphocytes developing from the transplanted cells during the growth of the tumor contained antibody. Hence, there was a transfer of immune globulin to daughter cells arising by mitosis from the labelled cells of the transplant. Moreover, the data show the presence of antibody in normal lymphocytes of the animals in this experiment. Inasmuch as there was no immune globulin present in these mice prior to their receiving the tumor transplant, the normal lymphocytes could have obtained the antibody only from malignant lymphocytes. These data taken together with those in Table I, Experiment 3, establish a reversible exchange of antibody protein between normal and malignant lymphocytes.

Discussion. This study establishes the presence of antibody in malignant lymphocytes.

In this respect, therefore, malignant lymphocytes of the particular type used resemble normal lymphocytes. In most of the experiments tumor cells contained more antibody per unit of nitrogen than did the normal lymphocytes. Also, the malignant cells were able to obtain significant quantities of antibody protein from normal lymphocytes.

The high degree of titer in tumors growing from transplanted, antibody-containing malignant lymphocytes indicates that labelled globulin is carried into the daughter cells. As the growth of the tumor proceeds, increasing numbers of antibody-containing lymphocytes are formed. Thus, the total quantity of available antibody in lymphoid structures is dependent upon the number of antibody-containing lymphocytes present. This type of antibody production, *i.e.*, multiplication of pre-existing cells containing immune globulin, may be a prominent mechanism of antibody production in the normal organism.

The data also show that antibody may be transferred from malignant to normal lymphocytes. This transfer may have occurred as a result of the liberation of immune globulin by degenerating, malignant lymphocytes. It is also possible that although evidence of metastases was not observed, some tumor cells may have infiltrated the normal lymphoid structures. This latter possibility is unlikely, however, since in the absence of gross metastases, it would be difficult to assume the presence of enough tumor cells in the normal lymphoid tissues to account for their antibody titers.

Summary. Staphylococcus antitoxin titers have been obtained from extracts of lymphocytes secured from a rapidly growing mouse lymphosarcoma. Comparison of these titers with those of the sera and normal lymphocytes of the same animals suggested that tumor tissue had a slightly higher antibody content. Tumor cells were capable of obtaining antibody from some other source in the body, presumably normal lymphocytes. The growth of an antibody-containing tumor transplant in normal mice was accompanied by the development of antibody-containing malignant cells. The normal lymphocytes of the host animal receiving this transplant contained antibody. There is a reversible exchange of antibody protein between normal and malignant lymphocytes.

15021

Sulfonamide Blood Levels Following Deposition Under the Bladder Peritoneal Reflection.

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Numerous reports on the rate of sulfonamide absorptions from various portions of the body have been published but information was not found on the blood levels following deposition under the bladder peritoneal reflection at the time of cesarean section operation.

In an effort to reduce the morbidity incidence, 5 g of a sulfonamide were placed above the uterine fascia just before the incised area was covered by suturing the bladder peritoneal reflection to the uterus. This tissue is essentially a loose areolar type. Blood levels were taken at 4-hour intervals from the fourth to the twenty-fourth hour basis following the cesarean section. Because of limited personnel and because the sixteenth and twentieth hours came late at night, only 2 tests for these times were made on the 32 patients.

Every patient had a test at the fourth hour while 30 had at the eighth and 26 at the twelfth hour, only 25 tests were completed at the twenty-fourth hour interval. Marshall's[1] method was used for these determinations.

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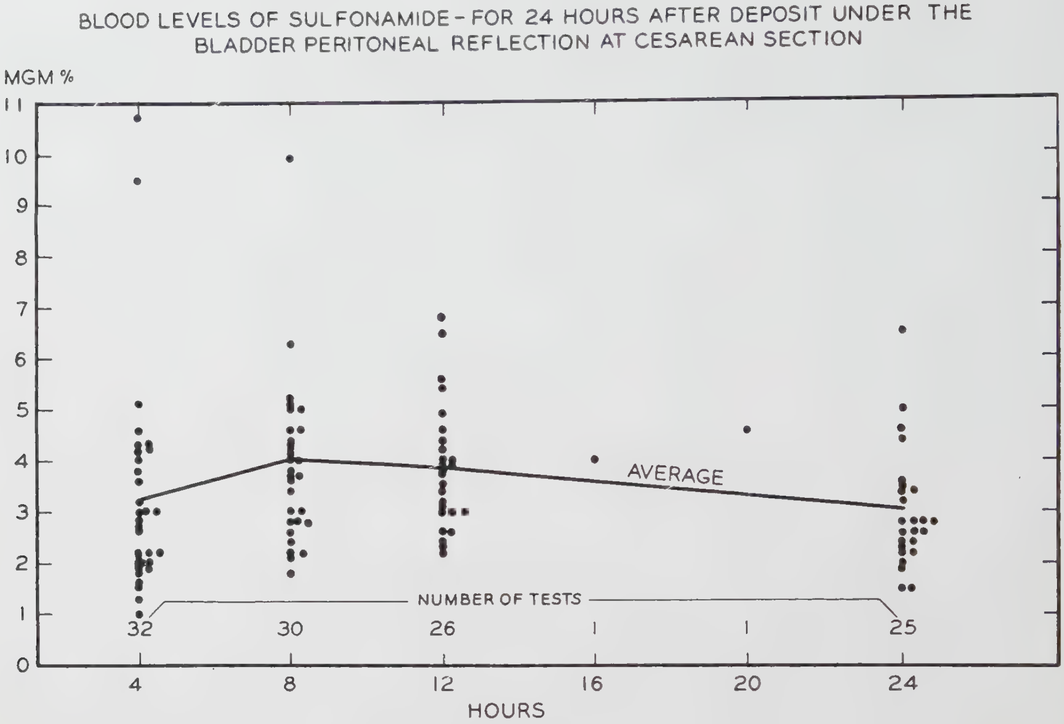
[1] Marshall, E. K., Jr., *J. Biol. Chem.*, 1937, **122**, 263; Marshall, E. K., Jr., Emerson, K., Jr., and Cutting, W. C., *J. A. M. A.*, 1937, **108**, 953.



The graph reveals the varied readings. Since there was no typical type of curve a mean average did not seem representative. The highest reading at the fourth hour dropped to 5.0 mg in 4 hours and to 4.4 by the twelfth hour, whereas one patient had a level of 9.5 in 4 hours and had a 9.9 at 8 hours.

Thirty of the 32 patients had 5 g of sulfanilamide placed under the peritoneal reflection. The remaining 2 had 5 g sulfathiazole. The readings of this latter drug were:

Hr	4th	8th	12th	16th	20th	24th
Mg	3.0	3.0	2.6	—	—	1.9
Mg	1.0	—	—	—	—	--

The blood levels of sulfanilamide and sulfathiazole were no doubt influenced by the variable fluid intake, the amount of tissue exudate, and other factors.

Conclusions. Blood levels following the placement of 5 g of sulfanilamide in 30 patients and 5 g sulfathiazole in 2 under the bladder peritoneal reflection did not exceed tolerance ranges. Ordinarily, good absorption can be expected from this site but the erratic and unpredicted rates make this an unsatisfactory route for establishing effective blood levels.

15022

Deformity of the Tongue Associated with Amino Acid Deficiencies in the Chick*

C. R. GRAU. (Introduced by H. J. Almquist.)

From the Division of Poultry Husbandry, University of California, Berkeley.

During the course of studies on the nutritive value of beef blood proteins,[1] it was observed that in some chicks being fed a diet deficient in isoleucine the distal end of the tongue was folded back onto the dorsal surface (Fig. 1). In some animals, it appeared superficially that the tip of the tongue had been cut off squarely, and it was only after loosening the tip from the surface with a probe that the true condition was seen. Microscopic examination of longitudinal sections of the tongue revealed that the limiting factor in the size of the turned-back portion was the length of the median cartilage. Subsequently, the same condition was observed in chicks fed diets deficient in leucine and phenylalanine. A search of the literature has not as yet revealed previous mention of this condition.

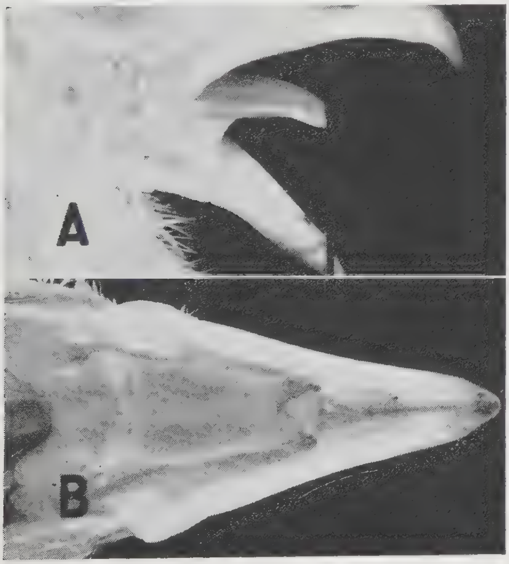


FIG. 1.
Gross appearance of affected tongue. A—Characteristic view showing squareness of tip. B—Dorsal view of tongue as it lies in the lower beak.

The chicks used were White Leghorns of 5 to 30 days of age at the start of the experiments, and all were reared on a chick starting mash from the day of hatching. The basal diet used in this study was composed of purified ingredients; its exact description (except for the protein source) has been described previously.[2]

The results obtained with various amino acid deficiencies are presented in Table I. All chicks which were fed the deficient diets lost weight during the experiments, while those receiving supplements gained during the same period. From the table it is seen that the incidence of the condition closely follows the isoleucine deficiency of the blood meal diets. The physical character of the diet is apparently not the controlling factor, because experiments in which a mixture of amino acids was used to replace all protein indicated that leucine and phenylalanine deficiencies are causative agents, while lack of histidine, valine or threonine shows no effect. When zein was used as the protein source, and was supplemented with all necessary amino acids except tryptophane, no cases were observed.

Since all of these cases were associated with weight loss, the possibility of inanition or secondary deficiency causing the folded tongues became apparent. Starvation of chicks whose initial weights were 160 grams resulted in death within a week, presumably too soon to allow manifestation of the lesion. Feeding 12 chicks a diet devoid of protein prolonged life, but no folded tongues occurred. Similar groups fed 1% or 2% sardine meal

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* This study was aided by grants from Swift and Company and The Nutrition Foundation, Inc. Crystalline biotin was kindly provided by Merck and Company.

[1] Grau, C. R., and Almquist, H. J., *Poultry Sci.*, 1944, **23**, 486.

[2] Almquist, H. J., and Grau, C. R., *J. Nutr.*, 1944, **28**, 325.

TABLE I.
Incidence of the Folded Tongue Deformity in Chicks.

Deficiency studied	Source of amino acids	Supplement	Duration of experiment, days	No. of chicks	Incidence, %
Isoleucine	Blood cell protein, 20%, + gelatin, 5%	None	12	4	50
,,	,, ,, ,,	*dl*-isoleucine, 2%	12	8	0
,,	,, ,, ,,	None	10	8	25
,,	,, ,, ,,	Alfalfa leaf meal, 20%	10	8	0
,,	,, ,, ,,	,, ,, ,, 10%	10	8	0
,,	,, ,, ,,	None	11	20	45
,,	,, ,, ,,	,,	15	15	33
Leucine	Amino acid mixture	,,	18	7	14
Phenylalanine	,, ,, ,,	,,	18	2	50
Histidine	,, ,, ,,	,,	19	4	0
Valine*	,, ,, ,,	,,	12	8	0
Threonine	,, ,, ,,	,,	14	4	0
Tryptophane	Zein + amino acids	,,	18	5	0

* All chicks fed this diet were dead by the 14th day.

protein lost slightly less weight during a 32-day period, and, with one exception, there were no cases of the folded-tongue condition. The single chick which showed the deformity was fed the 2% protein diet, and had been on the diet 16 days before the tongue became deformed. With a casein diet low in protein (2.0%) and supplemented with small amounts of arginine, glycine, and cystine, no cases were observed. It appeared from these results that causing a loss in weight does not necessarily bring on the condition.

Next, 3 proteins known to be deficient sources of amino acids were compared to see if any deficient protein source fed at the 20% protein level could cause the condition. The proteins chosen were dried whole beef blood, zein, and gelatin; the first is deficient primarily only in isoleucine, the second in glycine, lysine, and tryptophane, and the third in tryptophane, the sulfur amino acids, threonine, isoleucine, and probably other amino acids. There were 10 chicks per group at the start of the experiment, with an average weight of 83 grams. The chicks were placed on the diets and observed daily. After 12 days the average weight losses ranged from 7 grams (for the blood meal) to 18 grams (for the gelatin). The incidence of the folded-tongue syndrome and of mortality are apparently independent results of feeding these poor diets, for the mortality was essentially the same on all diets, while the folded-tongue incidence varied as follows (at 30 days): 90% for blood meal, 10% for zein, and 0% for gelatin. Edema was noted in some chicks on the blood-meal diets 1 to 3 days before death. This condition was most apparent in the tissue lying between the wattles.

Five chicks having folded tongues were placed on a chick starting ration which was known to be adequate for growth. After 9 days all tongues were apparently normal. When the birds were returned to the isoleucine-deficient diet previously used, no cases were observed at 7 days, but all had folded tongues at 13 days. These birds were again placed on the chick mash, and were observed frequently for the remainder of the experiment. There was no gross change for 4 days, but from the 5th day on, the folded portion gradually diminished in size until, by the 11th day, no trace could be seen.

Summary. A condition characterized by the folding-back of the tip of the tongue of chicks is described. A deficiency of isoleucine, leucine, or phenylalanine causes this syndrome, whereas deficiencies of protein or of several other amino acids have no effect. Gradual healing occurs about a week after chicks are placed on an adequate diet.

15023 P

Effect of Adjuvants on Vaccination of Human Beings Against Influenza.*

WERNER HENLE AND GERTRUDE HENLE.

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Immunization of human subjects against influenza Types A and B with adequate vaccines prepared from infected allantoic fluids of the chick have afforded protection against the natural or experimental disease for a limited period of time. In most instances the duration of the immune state has been found to correlate roughly with the height of the serum antibody level in the vaccinated individuals.[1] The titer reaches its peak about 2 weeks after vaccination and decreases thereafter more or less rapidly until the prevaccination level is reached, usually 4-12 months after immunization. This time relationship seems to hold regardless of the kind of vaccine used.[2] Therefore, the problem of protection against influenza lies not so much in the preparation and use of vaccines producing maximal antibody response in men, but rather in the maintenance of adequate antibody levels for a prolonged period of time.

The studies of Freund and his co-workers[3] have shown that the addition of certain adjuvants (lanolin-like substances and killed tubercle bacilli in paraffin oil) increase and prolong antibody production to various antigens in rabbits and guinea pigs. Friedewald[4] applied these findings to the immunization of animals against influenza. Recently Salk[5] reported the adjuvant effect of calcium phosphate on vaccination against influenza in mice. A study was begun in this laboratory in June, 1944, using influenza vaccines combined with adjuvants, except for the tubercle bacilli, in human beings and a brief summary of the results up to the sixth month is presented below.

Centrifugally concentrated influenza virus vaccine of Types A and B prepared according to the method of Stanley,[6] formed the stock material.[†] One part of this stock vaccine was emulsified in one part of Falba[‡] to which 4 parts of mineral oil[§] were added with constant agitation. Eighty female subjects received subcutaneously 0.3 ml of this vaccine (0.45 mg protein) either in a single or divided into 2 simultaneous injections. A similar group of 80 was injected with 0.3 ml of the same stock vaccine diluted 1:4 in buffered saline (0.7 mg of protein). All individuals were bled before vaccination and 2 weeks, 1, 2, 3, and 6 months thereafter. The sera were tested for their ability to inhibit chick red cell agglutination by influenza viruses A and B,[7] using a modification of Hirst's technic. The geometric mean antibody titers (initial serum dilution) were determined for the various groups. Since there was no difference between the subjects receiving the dose in one or 2 injections of

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[*] The work described in this paper was done under contract, recommended by the Committee on Medical Research, between the Office of Scientific Research and Development and the Children's Hospital of Philadelphia.

[1] Henle, W., Henle, G., and Stokes, J. Jr., *J. Immunol.*, 1943, **46**, 163.

[2] Hirst, G. K., Rickard, E. R., Whitman, L., and Horsfall, F. L., Jr., *J. Exp. Med.*, 1942, **75**, 495.

[3] Freund, J., and McDermott, K., PROC. SOC. EXP. BIOL. AND MED., 1942, **49**, 548; Freund, J., and Bonanto, M. V., *J. Immunol.*, 1944, **48**, 325.

[4] Friedewald, W. F., *J. Exp. Med.*, 1944, **80**, 477.

[5] Salk, J. E., *Science*, 1945, **101**, 122.

[6] Stanley, W. M., *J. Exp. Med.*, 1945, **81**, 193.

[†] The vaccine was prepared by Dr. B. Hampil of Sharp and Dohme, Inc., for the Committee on Medical Research. It contained per ml 2.5 mg each of the PR-8 and Weiss strains of influenza A and 4 mg of the Lee strain of influenza B virus.

[‡] Distributed by Pfalz and Bauer, Inc., New York.

[§] Atreol No. 9, of the Atlantic Refining Company, U. S. P. For further information see Halbert, S. P., Smolens, J., and Mudd, S., *J. Immunol.*, in press.

[7] Hirst, G. K., *J. Exp. Med.*, 1942, **75**, 49.

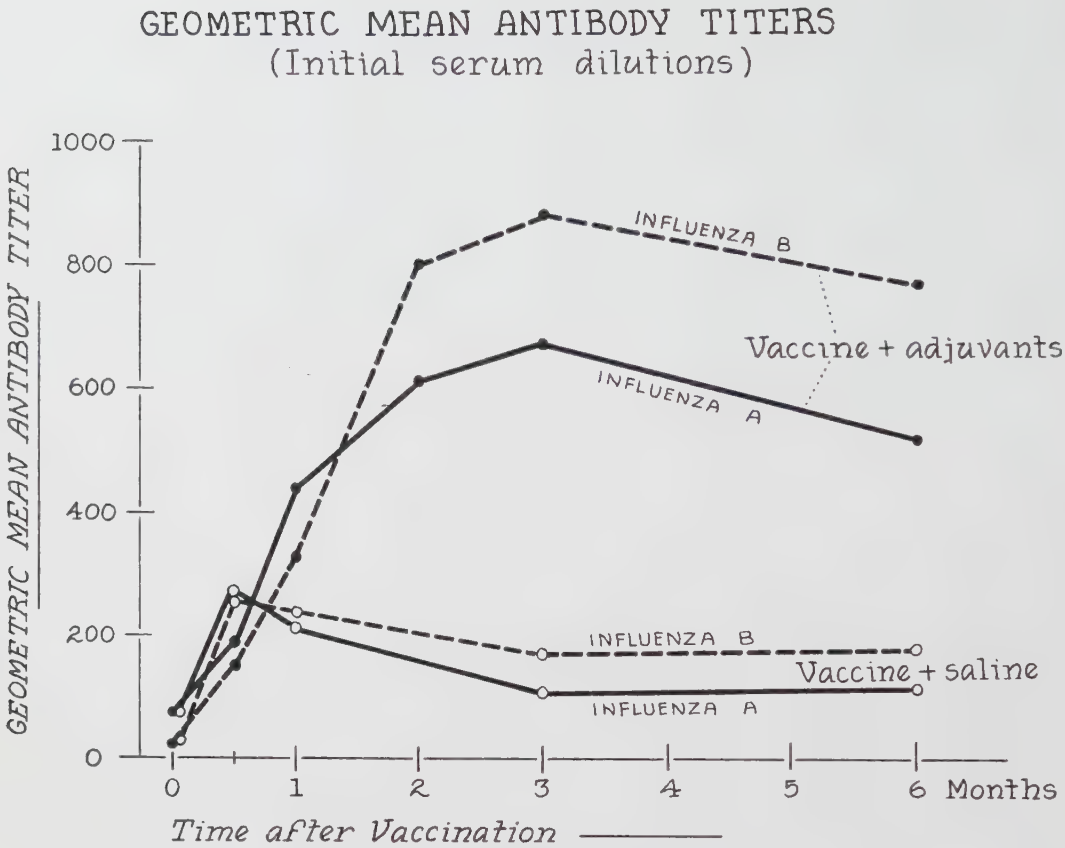

FIG. 1.
Geometric mean antibody levels over a period of 6 months following vaccination.

the Falba-mineral oil vaccine (FMV) the data of the two groups were combined. The results for the FMV and the saline vaccine (SV) series are presented in Fig. 1.

The titers in the SV series reached their peak in 2 weeks and fell gradually thereafter. In the FMV group the 2-weeks-result was inferior to that of the SV series but the titer continued to rise and reached its maximum in 2 to 3 months and only slight decreases were noted at the 6-month interval. The maxima of the FMV series were 2.5 to 3 times higher than the maxima of the SV series. By the time the FMV group reached its maximum the geometric mean antibody level was about 5 to 6 times higher than in the SV series at the corresponding time (3 months) and after 6 months the difference was still 4 to 5 fold.

Practically all individuals in the FMV series had antibody titers of more than 128 against both influenza A and B viruses and 93% and 96% respectively were still in this group at the end of 6 months. On the other hand in the SV series at this time 66% and 52% respectively had titers below this level. On the basis of experimental studies[1] one may consider individuals with titers of more than 128 as probably immune, those with lower levels as potentially susceptible. Sufficient data on this point regarding epidemics are lacking.

These results show that addition of Falba and mineral oil to influenza vaccines improves the antibody response in human beings and keeps the antibodies at a high level as compared with the results of vaccination with a slightly larger amount of antigen suspended in buffered saline. The technic of injection deserves further study and the optimal total and relative amounts of adjuvants and vaccine need to be determined. Nodules of varying dimensions (up to 3 cm diameter) could be palpated in the subcutaneous tissues of most individuals. These decreased gradually in size and were still evident in roughly 40% of the cases 6 months after vaccination. In 2 cases abscesses were noted about 2 months after the injection, one of which had to be drained surgically. This number among 120 sites of injection is not too discouraging in the light of the early experience with alum precipitated toxoids.

15024 P

Effect of Amino Acids on Acetylcholine Synthesis.*

Clara Torda and Harold G. Wolff.
From the New York Hospital and the Departments of Medicine (Neurology) and Psychiatry, Cornell University Medical College, New York City.

In the presence of serum and spinal fluid more acetylcholine is synthesized by the frog brain *in vitro* than in the absence of these body fluids.[1,2,3] In search for the agents responsible for the greater acetylcholine synthesis the effect of amino acids was investigated.

Experimental. The effect of the substances used on the acetylcholine synthesis was studied by the method described previously.[2] Mixtures containing varying amounts of the substances, 100 mg minced fresh frog brain, 3 mg physostigmine salicylate, 4.8 mg glucose, and 3 cc Ringer's solution were shaken and incubated aerobically for 4 hours at 37°C. After incubation the amounts of acetylcholine synthesized were assayed biologically on the sensitized rectus abdominis muscle of the frog.

The amounts of acetylcholine synthesized are given in Table I. Most of the amino acids in the concentrations used (10^{-2} to 10^{-5} mol.) increased the amount of acetylcholine synthesized. The naturally occurring form of the amino acids seemed to increase the synthesis more than the naturally not occurring form. Nachmansohn and John[4] under different experimental conditions (different enzyme preparation, different substrate, and anaerobically) found a similar increase of the amount of acetylcholine synthesized in the presence of *l* (+) glutamic acid, cysteine, alanine, phenylalanine, *l* (—) tryptophane, *l* (—) proline, and valine.

It was also found[5] that acetylcholine synthesis in the presence of serum from the working arm was 40% less than in the presence of serum from the immobilized arm. Since it is possible that during muscle work some transamination occurs[6] the effect of free ammonia on the synthesis of acetylcholine was also investigated. It was found that ammonia decreased the acetylcholine synthesis. Therefore, if ammonia accumulates during performance of muscle work it may depress the local acetylcholine synthesis, a synthesis known to occur during performance of prolonged work by cholinergic systems.[7,8] The accumulation of ammonia may thus contribute to the genesis of muscle fatigue.

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* This investigation was aided by a grant from the John and Mary R. Markle Foundation.

[1] Torda, C., and Wolff, H. G., *Science,* 1943, **98**, 242.

[2] Torda, C., and Wolff, H. G., *J. Clin. Invest.,* 1944, **23**, 649.

[3] Torda, C., and Wolff, H. G., *Science,* 1944, **100**, 200.

[4] Nachmansohn, D., and John, H. M., *J. Biol. Chem.,* 1945, **158**, 157.

[5] Torda, C., and Wolff, H. G., Proc. Soc. Exp. Biol. and Med., 1945, **59**, 13.

[6] Cohen, *Wisconsin Symposium on Respiratory Enzymes,* 1942, p. 210.

[7] Brown, G. L., and Feldberg, W., *J. Physiol.,* 1936, **88**, 265.

[8] Kahlson, G., and MacIntosh, F. C., *J. Physiol.,* 1939, **96**, 277.

TABLE I.
Effect of Amino Acids on the Synthesis of Acetylcholine.

	Amt of acetylcholine synthesized in % of control†				
	Amt of substances (mg) added to 100 mg frog brain				
Substance*	30	3	0.3	0.03	0.003
I. Aliphatic Amino Acids					
(A) Monoamino monocarboxylic acids:					
glycine (SMACO)		98	103	101	102
dl-alanine (Fischer)		116	117	113	104
dl-serine (Dr. duVigneaud)		119	126	110	100
dl-threonine (SMACO)		117	115	116	112
l(—)leucine (Dr. duVigneaud)		106	104	105	103
d(+)leucine (Dr. duVigneaud)		111	111	105	98
(B) Diamino monocarboxylic acids:					
l(+)arginine (Pfanstiehl)		140	129	120	100
l(+)arginine (SMACO)		128	125	115	105
l(+)lysine monohydrochloride (SMACO)		123	112	107	103
l(+)lysine dihydrochloride (Eastman)		136	128	123	110
dl-lysine dihydrochloride (Eastman)		109	108	106	105
(C) Sulfur-containing amino acids:					
l(—)cystine (Fischer)		123	117	107	100
l(—)cystine (Dr. duVigneaud)		126	115	110	103
dl-methionine (SMACO)		102	99	103	99
l(—)methionine (Dr. duVigneaud)		145	129	113	103
l(—)cysteine (SMACO)		134	135	130	112
l(—)homocystine (Dr. duVigneaud)		120	110	114	98
II. Aromatic Amino Acids)					
dl-phenylalanine (SMACO)		110	114	102	105
l(—)tyrosine (Fischer)		112	110	111	104
III. Heterocyclic Amino Acids					
dl-histidine (Fischer)		107	106	108	104
d(+)histidine (Dr. duVigneaud)		134	120	108	102
l(—)histidine (Dr. duVigneaud)		142	125	115	103
l(—)tryptophane (Merck)		132	114	117	103
dl-tryptophane (Dr. duVigneaud)		123	116	114	94
dl-tryptophane (SMACO)		107	105	103	98
tyroxine (Squibb)			133	113	113
Dipeptide: carnosine (Dr. duVigneaud)		160	136	121	100
Tripeptide: glutathione (Fischer)			128	124	114
Decomposition Products:					
creatine		121	116	108	104
creatinine		103	97	101	98
ammonia	15	34	67	90	101

* The substances prepared by Dr. duVigneaud were pure amino acids, the other amino acids may have contained impurities.

† Each value represents the average of 8 separate experiments. The S.E. of the mean for each value was less than ±5%. The amount of acetylcholine synthesized in μg per 100 mg frog brain, followed by the S.E. of the mean, was: 1.50 ± 0.044.

Discussion. The increase of the synthesis of acetylcholine in the presence of amino acids is probably due to various factors: (1) the ability of amino acids to remove heavy metals (such as Cu); (2) to the property of some amino acids to re-establish the free –SH group, an active group of the enzyme involved in the synthesis of acetylcholine; and (3) other not yet identified mechanisms. The $-CH \cdot NH_2 \cdot COOH$ group is partly responsible for the increase of acetylcholine synthesis since greater increase was observed in the presence of substances containing 2 or more $-NH_2$ group (diaminomonocarboxylic acids, peptides) than in the presence of the amino acids containing only one $-NH_2$ group; and phenylalanine and tryptophane increased the synthesis while phenol and indol decrease it.[9]

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[9] Torda, C., and Wolff, H. G., PROC. SOC. EXP. BIOL. AND MED., 1945, **59**, 183.

It seems that the ketogenic amino acids increased the acetylcholine synthesis less than the non-ketogenic amino acids.

Summary. 1. The effect of amino acids, some polypeptides, and a few decomposition products on the synthesis of acetylcholine was investigated. 2. Most of the amino acids, carnosine, glutathione, and creatine increased the synthesis. 3. Glycine, leucine, and creatinine did not modify the synthesis. 4. Ammonia decreased the amount of acetylcholine synthesized.

15025 P

Effect of Some Isocyclic, Aromatic, and Heterocyclic Compounds on Acetylcholine Synthesis.*

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In the presence of body fluids of patients with myasthenia gravis less acetylcholine is synthesized than in the presence of body fluids of healthy subjects.[1,2,3] In the following it was ascertained whether some isocyclic, aromatic hydrocarbons, and heterocyclic compounds modify the synthesis of acetylcholine. These substances were chosen because some of them occur in the animal organism as intermediate or end product of the metabolism, some are widely administered for their therapeutic effect, and some are known to modify the activity of various enzymes.

Experimental. The effect of the substances on the synthesis of acetylcholine was studied by the method described previously.[2] The amounts of acetylcholine synthesized are given in Table I. Isocyclic (cyclohexane, inositol) and aromatic hydrocarbons (benzol, toluol, benzoic acid, naphthalene) either did not modify the synthesis of acetylcholine or decreased it only in higher concentrations (10^{-2} mols). Hydroxyl derivatives (phenol, *p*-aminophenol, hydroquinone, salicylic acid, α-naphthol, β-naphthol), aldehydes (benzaldehyde), and ketones (camphor, penicillin) decreased the synthesis in concentrations from 10^{-5} mol. Conjugated products either did not modify the synthesis or decreased it slightly (potassium phenol sulfonate, phenacetine, diphenylamine, benzidine, acetylsalicylic acid, sulfonamides). From the heterocyclic compounds indol decreased the synthesis in concentrations from 10^{-5} mol. Substitution in β position decreased this property of indol: skatol was somewhat less active depressor, indol-3-acetic acid was nearly inactive, *dl*-tryptophane was inactive, and *l* (—) tryptophane, the naturally occurring amino acid, increased the ability of frog brain to synthesize acetylcholine. Quinoline decreased the synthesis in concentrations from 10^{-5} mol.; carbazole decreased it to a lesser degree.

Discussion. The substances used differ in their chemical structure and their ability to enter into various chemical reactions but are known to inactivate proteins. Therefore, it is possible that the decrease of acetylcholine synthesis was due to a partial inactivation of the protein component of the enzyme involved in the synthesis. Many of the substances used are readily combined in the animal organism forming less toxic compounds. Conjugated substances depressed the synthesis of acetylcholine to a lesser degree than did the corresponding acids.

A correlation between the decrease of synthesis of acetylcholine *in vitro* and the physio-

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* Aided by a grant from the John and Mary R. Markle Foundation.

[1] Torda, C., and Wolff, H. G., *Science*, 1943, **98**, 242.

[2] Torda, C., and Wolff, H. G., *J. Clin. Invest.*, 1944, **23**, 649.

[3] Torda, C., and Wolff, H. G., *Science*, 1944, **100**, 200.

TABLE I.
Effect of the Substances Used on Synthesis of Acetylcholine.

		Amt of total acetylcholine synthesized in % of control*			
		Amt (mg) of substances added to 100 mg frog brain			
Substance	No. of exp.	3	0.3	0.03	0.003
Inositol	11	98	99	98	105
Cyclohexane	8	88	98	100	101
Benzol	10	84	94	97	98
Toluol	7	109	108	100	104
Benzoic acid	8	80	90	96	103
Benzaldehyde	8	44	65	77	95
m-dinitrobenzene	6	58	75	80	90
Phenol	9	30	55	77	111
Potassium phenol sulfonate	8	104	97	99	102
Phenacetin	8	66	103	105	99
Sulfanilamide	8	113	117	114	107
Sulfapyridine	8	109	106	104	105
Sulfathiazole	8	100	106	107	104
Sulfadiazine	8	99	100	96	97
Diphenylamine	8	80	105	112	114
Benzidine HCl	8	67	97	105	106
p-aminophenol	9	52	69	111	109
Hydroquinone	10	40	71	98	98
Salicylic acid	9	35	67	79	87
Acetylsalicylic acid	9	113	105	105	102
Naphthalene	8	82	97	104	106
α-naphthol	8	21	50	81	93
β-naphthol	8	22	50	84	100
Quinoline	8	54	66	76	83
Indol	9	41	48	88	95
Skatol	8	51	57	88	99
Indol-3-acetic acid	7	75	100	103	103
dl-tryptophane	10	108	105	102	98
l(—)tryptophane	11	132	114	117	103
Carbazole	9	82	87	92	99
Camphor	11	31	78	96	105
		Amt of penicillin added in Oxford units			
		15,000	1,500	150	15
Penicillin (Merck)	5	15	58	90	94

* The S.E. of the mean for each value was less than ±5%. The amount of acetylcholine synthesized in μg per 100 mg frog brain, followed by the S.E. of the mean, was 1.50 ± 0.044.

logical effects of the substances in the living animal is not yet possible. However, symptoms occurring during intoxication by phenol, salicylic acid, camphor, and benzol have been described as resembling the symptoms occurring during atropine poisoning.[4] It is conceivable that some of the symptoms are due to a lack of acetylcholine.

Sulfonamides and acetylsalicylic acid, in the concentrations used, did not modify the synthesis of acetylcholine. Penicillin, an unsaturated ketone, depressed the synthesis probably because of its ability to react with the sulfhydryl group,[5] an active group of the enzyme involved in the synthesis of acetylcholine.

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[4] Goodman, L., and Gilman, A., *The Pharmacological Bases of Therapeutics*, Macmillan Co., New York, 1941.

[5] Geiger, W. B., and Conn, J. F., *J. Am. Chem. Soc.*, 1945, **67**, 112.

15026

In vitro Effects of Specific Immune Blood on Certain Blood and Tissue Flagellates.*

Harry A. Senekjie and Ruth A. Lewis. (Introduced by E. C. Faust.)

From the Department of Tropical Medicine, Tulane University of Louisiana, New Orleans, La., in cooperation with the Tulane Amebiasis Unit of the National Institute of Health.

In a study of the *in vitro* effects of specific immune sera upon several species of *Leishmania,* Kligler[1] found that when high concentrations of the serum are added to cultures, the homologous organisms are lysed while the heterologous organisms are not affected. He further noted that the exposure of cultures of organisms to dilute homologous sera caused them to agglutinate but did not inhibit their growth.

Malamos[2] found that the addition of immune serum to cultures of the *Leishmania* tended to retard their growth to some extent, but that immune serum did not have any greater action on homologous organisms than on other species of *Leishmania.*

In the course of our investigations on the antigenic composition of the blood and tissue flagellates it was observed that when young, actively growing cultures of *Trypanosoma cruzi* were added to a culture medium which contained homologous immune blood the organisms did not survive.

To obtain a clearer understanding of this reaction a series of experiments was planned (1) to determine the extent to which this inhibitory or lethal effect of immune blood is consistently associated with the various species of blood and tissue flagellates, (2) the specificity of the reaction (3) and the nature of the factor or factors responsible for this phenomenon.

Materials and Methods. Immunization of Rabbits. Rabbits were immunized with killed cultures and with living cultures of *L. brasiliensis, L. donovani, L. tropica,* and *T. cruzi.* Antigens were prepared according to the method described by Senekjie[3] and were administered by a schedule which has been previously outlined (Senekjie and Lewis[4]). One week after the agglutinin titers had risen sufficiently the animals were bled, employing strict aseptic technics. From 7 to 10 cc of blood were collected into each of 2 tubes, one of which contained 1 cc of sterile 3.5% sodium citrate.

Blood was similarly collected from normal rabbits.

Preparation of Media. 1. *Immune whole blood leishmania agar medium.* To different batches of leishmania agar (Senekjie[3]) at 45°C, sufficient immune citrated blood of rabbits immunized with antigens of *L. brasiliensis, L. donovani, L. tropica,* and *T. cruzi* was added to make 6 to 9% of the respective media. The media were tubed and made into slants.

One set of each medium was preheated in a water bath at 56°C for 30 minutes, another set at 70°C for 30 minutes, and the remaining sets were not heated.

2. *Immune erythrocyte-normal serum leishmania agar medium.* Erythrocytes of the rabbits immunized with antigens of *L. donovani* were washed 4 times in sterile physiological salt solution. The washed cells were then resuspended in an equal volume of normal rabbit serum. Ten cc of this mixture

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[1] Kligler, I. J., *Tr. Roy. Soc. Trop. Med. and Hyg.,* 1925-26, **19**, 330.

[2] Malamos, B., *Arch. f. Schiffs. u. Tropen-Hyg.,* 1937, **41**, 416.

[3] Senekjie, H. A., Proc. Soc. Exp. Biol. and Med., 1943, **52**, 56.

[4] Senekjie, H. A., and Lewis, R. A., Proc. Soc. Exp. Biol. and Med., 1944, **57**, 17; *Am. J. Trop. Med.,* in press.

were added to 90 cc of leishmania agar at 45°C. The medium was then tubed and slanted. Similarly, medium was prepared with the washed erythrocytes of rabbits immunized with *T. cruzi* antigens.

3. *Immune serum-normal erythrocyte leishmania agar medium.* Media were prepared with the serum of rabbits immunized with *L. donovani* and *T. cruzi* antigens. Five cc of the respective immune sera were mixed with 5 cc of washed erythrocytes of normal rabbits and each mixture was added to 90 cc of leishmania agar at 45°C. The media were put into tubes and slanted.

4. *Non-immune whole blood leishmania agar medium.* To leishmania agar at 45°C sufficient citrated or defibrinated normal rabbit blood was added to make 10% of the medium, which was then dispensed in tubes and made into slants.

General Methods. All media were incubated at 37°C for a period of 24 hours to determine bacterial sterility. Prior to inoculation each tube was overlaid with 1 cc of sterile physiological salt solution. The inoculum in each case was 0.25 cc of a rich suspension of the flagellates from cultures 3 to 5 days old. The effects of various constituents of immune blood were studied in a series of experiments by inoculating a single species of flagellate into several tubes of culture media, of which one tube contained the homologous immune material, and each of the other tubes contained a particular type of heterologous immune substance. For example, *L. brasiliensis* was inoculated into a medium containing homologous immune material and into other media containing anti-donovani, anti-tropica, and anti-cruzi immune material respectively, and a similar procedure was employed for *L. donovani, L. tropica* and *T. cruzi.* The experiments were run in duplicate using blood from 2 rabbits similarly immunized, and each experiment was repeated 3 times. Microscopic examinations of the unstained cultures were made at intervals of 1 to 2 days. Morphological changes and the amount of growth were noted. When viable organisms could not be demonstrated by microscopic examination, subcultures were made on the regular leishmania blood agar medium as an additional test of viability.

The control culture medium in these experiments consisted of non-immune whole rabbit blood in leishmania agar, with an overlay of physiological saline.

Results. Effect of immune whole blood in leishmania agar medium. In these experiments the different tubes of media contained a particular kind of immune whole blood. Following the inoculation of these media with each species of organism the cultures were examined over a period of 3 weeks at intervals of 1, 2, 2, 2, 3, 7, and 4 days. The results observed in the duplicate experiments were practically identical in each instance.

The effects of unheated immune whole blood were studied first. These experiments revealed that when the organisms were grown in the homologous immune blood media they showed varying degrees of inhibition depending upon the species, while those grown on heterologous immune blood media manifested no inhibition. The propagation of *L. donovani* was almost completely inhibited from the first day while *T. cruzi* showed a complete inhibition after 24 hours. The organisms first lost their motility, then became agglutinated, swelled to 2 to 5 times their normal size, rounded up, and finally developed complete hyalinization of the internal structure, so that the nucleus, parabasal body, axoneme, undulating membrane or flagellum could not be seen. In the case of *L. donovani* this phenomenon was very marked but less so than in the case of *T. cruzi.* When such forms were placed on the regular leishmania blood agar medium, no propagation was obtained, indicating that the organisms had been killed. *L. brasiliensis* and *L. tropica* on the homologous immune blood media showed a moderate inhibition over a period of 2 weeks. This reaction was most noticeable in the first 2 to 4 days. Such organisms had a tendency to agglutinate and were not actively motile, but remained viable as long as organisms grown on the heterologous immune blood media.

Following the demonstration of the inhibitory or lethal effect of unheated homologous immune blood on certain species of organisms, a series of experiments was carried out which was designed to elucidate the factors responsible for this action. These included an evaluation of the role of complement, studies on the thermostability of the inhibitory factors, and the determination of whether the factors are contained in the erythrocytes or the serum of immunized animals.

In the experiments to determine whether complement is necessary for the inhibitory or lethal effect of immune blood, the media containing immune whole blood were pre-heated at 56°C for 30 minutes. The results observed in these experiments were comparable in every respect to those encountered when unheated immune blood was used. Thus, complement was found to be unnecessary in the production of the inhibitory or lethal effect.

The preceding experiments demonstrated that the inhibitory or lethal factors are not destroyed by pre-heating at 56°C for 30 minutes. The thermostability of these factors was also determined by pre-heating the homologous immune whole blood medium at 70°C for 30 minutes. When this was done *L. donovani* survived for 2 weeks although the growth was poor, while *T. cruzi* remained viable for only 4-7 days. However, *L. brasiliensis* and *L. tropica* grew more abundantly than on the same medium, unheated or pre-heated at 56°C, but definitely less than in the control medium containing normal blood. These results indicate that the inhibitory or lethal factors are partially stable at 70°C. Unfortunately, the thermostability could not be tested at higher temperatures because of the danger of inactivating the growth-promoting factors. These factors are present in the serum and are known to be inactivated at 100°C. (Senekjie and Lewis[4]).

Effects of immune serum-normal erythrocytes and immune erythrocytes-normal serum in leishmania agar medium. In these experiments the distribution of the inhibitory or lethal factors in the blood was studied. This was done by inoculating *L. donovani* and *T. cruzi* into media containing homologous immune serum and normal erythrocytes, and into medium containing homologous immune erythrocytes and normal serum. The growth of *L. donovani* and *T. cruzi* was completely inhibited when the medium contained the homologous immune serum and normal erythrocytes. However, growth was normal and abundant when they were inoculated on a medium containing immune erythrocytes and normal serum.

Discussion. When various blood and tissue flagellates are grown in a medium containing homologous immune whole blood or serum, a distinct inhibitory or lethal action occurs in cultures of *T. cruzi* and *L. donovani,* and a less marked action occurs in those of *L. brasiliensis* and *L. tropica.*

The factors responsible for this inhibitory or lethal effect are not clearly understood. The present study indicates that they are (1) specific in their action, (2) present in immune serum but absent in immune erythrocytes, (3) absent in non-immune blood, (4) able to act in the absence of complement, and (5) partially inactivated by being heated at 70°C for 30 minutes. The most likely hypothesis is that the effect is due to such humoral factors as antibodies.

If the effect is due to antibodies, these observations have much fundamental significance, since they demonstrate a type of lytic antigen-antibody reaction that does not depend on the presence of complement. Complement is essential in bactericidal and hemolytic reactions. Although complement-fixing antibodies are produced in various protozoan infections (see Culbertson[5] for a review), the present study indicates that complement is not essential for the action *in vitro* of these inhibitory or lethal factors. Moreover, this effect is apparently not due to ablastin, since this antibody does not exert its effect *in vitro* (Taliaferro[6,7]).

Summary and Conclusions. The blood of rabbits immunized with various blood and tissue flagellates has been found to exert an inhibitory or lethal action on cultures of the homologous organisms. This effect was very marked on cultures of *Trypanosoma cruzi* and *Leishmania donovani,* but was slight on those of *L. brasiliensis* and *L. tropica.* These factors are associated with the blood serum, are thermostable at 56°C, but are partially destroyed at 70°C.

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[5] Culbertson, J. T., *Immunity Against Animal Parasites,* Columbia University Press, New York, 1941.

[6] Taliaferro, W. H., *J. Exp. Med.,* 1924, **39**, 171.

[7] Taliaferro, W. H., *Am. J. Hyg.,* 1932, **16**, 32.

The inhibitory or lethal action is observed *in vitro* in a medium prepared with the immune whole blood or immune serum on which the homologous organisms are grown. The reaction takes place in 3 stages, viz.,

(1) The organisms lose their motility and become agglutinated.

(2) They round up and become markedly swollen.

(3) Finally, the internal structure undergoes hyalinization which ends in the death of the protozoon.

Complement is not necessary for this reaction. This inhibitory or lethal factor appears to differ from ablastin.

15027

Penicillin Inhibitor from Staphylococci Which Have Developed Resistance to Penicillin in the Human Body.*†

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In previous reports[1,2] from this laboratory it was pointed out that strains of coagulase-positive staphylococci from human patients varied in their *in vitro* sensitivity to penicillin. None of these naturally resistant strains had been previously exposed to penicillin. Three strains of staphylococcus were made highly resistant to penicillin by exposing the organisms to increasing concentrations of penicillin. At that time, it appeared that acquired *in vitro* resistance to penicillin, like acquired sulfonamide resistance, was probably a permanent characteristic of these strains of staphylococcus. These resistant strains were subcultured on veal infusion agar slants every 3 to 4 weeks, and it was surprising to observe that within a year, the property of penicillin insensitivity had been completely lost. This was in contrast to 2 strains which had become resistant in patients as a result of penicillin therapy and still retained their resistance to penicillin after having been handled and subcultured in the same manner. The observations prompted further investigations to determine if staphylococci made resistant to penicillin by exposure *in vitro* differed from strains which had become resistant in the human body following the administration of penicillin.

Previous investigators[3] had shown that an enzyme-like substance called penicillinase, and extracted from *E. coli,* inhibited the action of penicillin. However, such an inhibitor could not be obtained from at least 6 strains of staphylococcus which were sensitive to penicillin, nor from 2 strains of staphylococcus which had been made resistant to penicillin *in vitro*.[4,5,6] Kirby,[7] employing a meth-

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† The penicillin was provided by the Office of Scientific Research and Development from supplies assigned by the Committee on Medical Research for investigations recommended by the Committee on Chemotherapeutic and Other Agents of the National Research Council.

[1] Spink, W. W., Ferris, V., and Vivino, J. J., PROC. SOC. EXP. BIOL. AND MED., 1944, **55**, 207.

[2] Spink, W. W., Ferris, V., and Vivino, J. J., PROC. SOC. EXP. BIOL. AND MED., 1944, **55**, 210.

[3] Abraham, E. P., and Chain, E., *Nature,* 1940, **146**, 837.

[4] Abraham, E. P., Chain, E., Fletcher, C. M., Gardner, A. D., Heatley, N. G., and Jennings, M. A., *Lancet,* 1941, **2**, 177.

[5] Bondi, A., and Dietz, C. C., PROC. SOC. EXP. BIOL. AND MED., 1944, **56**, 132.

[6] Bondi, A., and Dietz, C. C., PROC. SOC. EXP. BIOL. AND MED., 1944, **56**, 135.

[7] Kirby, W. M. M., *Science,* 1944, **99**, 452.

od described by Harper[8] for paracolon bacilli, extracted with acetone and ether 7 strains of coagulase-positive staphylococci which were naturally resistant to penicillin, and reported that the dried bacterial residue contained a potent inactivator for penicillin. He could not obtain inactivator from 7 coagulase-positive sensitive strains. Kirby concluded that the inactivator from staphylococci destroyed penicillin.

No reports have appeared concerning the presence or absence of penicillin inhibitor in staphylococci which had become resistant to penicillin in the human body following administration of the drug. The following results show that extracted staphylococci, which became resistant to penicillin in the body, possess an inhibitor for penicillin. This is in contrast to the absence of demonstrable inhibitor in parent-sensitive strains which were isolated before therapy was instituted. Furthermore, staphylococci which had developed a high degree of resistance to penicillin following exposure *in vitro* to increasing concentrations of penicillin did not produce demonstrable inhibitor.

Methods of Study. Source of strains. Sixteen strains of coagulase-positive staphylococcus, including 4 strains which became resistant to penicillin *in vivo,* and their 4 parent strains which were sensitive, were studied. Strains 22J and 23B were supplied to us through the courtesy of Dr. Donald Anderson of Boston. The parent strains of these cultures were isolated from patients with chronic osteomyelitis. The penicillin-resistant organisms were recovered from the same patients after penicillin had been administered. Strains 22J and 23B have retained their resistance on culture medium for over 2 years. Strain Long was cultured from the infected operative wound of a patient following nephrectomy. Cultures after penicillin therapy revealed the presence of insensitive staphylococci. The strain Eden was isolated from a patient with chronic osteomyelitis. This patient had been treated with sulfathiazole, and then penicillin, and subsequent cultures showed the presence of cocci which were resistant to both sulfathiazole and penicillin. Strain Rutgers was obtained from a skin lesion, while strains Ked and Nelson were isolated from patients with chronic osteomyelitis. These 3 strains along with Eden II were sensitive to penicillin.

Development of in vitro resistance. Strains Rutgers, Ked, Nelson, and Eden II were made resistant to sodium penicillin by exposing the organisms in Gladstone's medium[9] to increasing concentrations of the same lot of commercial penicillin. Similar results were obtained when veal infusion broth was used. Briefly, the technic was as follows: each of several tubes contained 9 cc of the medium to which was added 1 cc of freshly prepared sodium penicillin dissolved in isotonic saline solution. A standard loopful of a 24-hour culture of organisms in liquid medium was seeded to medium containing the added penicillin in a concentration which permitted demonstrable growth at 37°C within 24 hours. Loopful transfers of the organisms were made every 24 to 48 hours in a given concentration of penicillin until maximum growth could be attained within 24 hours. Then the concentration of penicillin was increased and the serial transfers repeated. In this manner, 100 to 200 transfers were made, and the resistance attained was related to the number of transfers.

In vitro test for penicillin sensitivity. From 100,000 to 300,000 organisms of a 24-hour culture of each strain were seeded into a series of test tubes containing Gladstone's medium. Freshly prepared aqueous solutions of sodium penicillin were added in varying concentrations to the resulting bacterial suspensions. The final volume in each tube was 10 cc. Incubation was carried out for 48 hours at 37°C. Inhibition of bacterial growth was ascertained by the absence of turbidity, and the end point determined by selecting that tube which remained clear with the lowest concentration of penicillin.

Detection of penicillin inhibitor. Each strain of staphylococcus was extracted with acetone and ether according to Harper's method.[8] Sterile suspensions of the dried extracted bacteria in physiologic saline solution were added in varying amounts to solutions

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[8] Harper, G. J., *Lancet,* 1943, **2**, 569.

[9] Spink, W. W., and Vivino, J. J., *J. Clin. Invest.,* 1944, **23**, 267.

TABLE I.
Relation Between Resistance to Penicillin and Production of an Inhibitor.
A. Strains Developing Resistance to Penicillin in the Human Body.

	Parent strains (Control)		Resistant variant-strains	
Strain	Oxford units penicillin per cc inhibiting growth	Presence or absence of penicillin inhibitor	Oxford units penicillin per cc inhibiting growth	Presence or absence of penicillin inhibitor
22J	0.05	Absent	1.0	Present
23B	0.05	”	2.0	”
Long	0.05	”	1.0	”
Eden	0.1	”	0.8	”
B. Strains Developing Resistance to Penicillin *in vitro*.				
Rutgers	0.2	Absent	40.	Absent
Ked	0.2	”	200.	”
Nelson	0.2	”	10.	”
Eden II	0.2	”	20.	”

of commercial penicillin in culture medium containing a standardized inoculum of penicillin-sensitive staphylococci. After incubation at 37°C for 48 hours, the presence or absence of viable bacteria was determined by culturing the contents of each tube on blood agar plates.

Results. In Table I is presented a summary of findings concerning the presence or absence of penicillin inhibitor in the extracted organisms of the 16 strains of staphylococcus studied. The significant feature is that an inhibitor of penicillin was obtained only from those 4 strains which had developed resistance to penicillin in the human body. No penicillin inhibitor was demonstrated in the dried, extracted bacteria of the remaining 12 strains even in a concentration of 1 mg of the dried bacteria per cc of penicillin solution. The extracted bacteria of strains which yielded inhibitor produced varying degrees of penicillin inactivation. The penicillin-resistant strain 23B produced the most potent inhibitor. A concentration of 0.1 mg of dried bacteria inhibited the action of 400 units of sodium penicillin per cc.

Summary. Strains of staphylococci which have developed resistance to penicillin in the human body yield an inhibitor for penicillin. Penicillin inhibitor was not present in 4 strains which had been made highly resistant by exposure *in vitro*. Resistance developed *in vivo* was found, among the strains tested, to be a more permanent characteristic of staphylococci than that acquired *in vitro*.

15028 P

Effect of Pituitary Implants and of Estradiol on Newly Metamorphosed Frogs, *Rana pipiens*.

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Primary and secondary sex organs respond markedly to treatment with hormones. It has been shown that the treatment of some immature salamanders with pituitary extracts results in marked testicular hypertrophy and increased spermatogenesis.[1] Also, other experiments have demonstrated that some estrogenic substances cause increased growth of Mullerian ducts in the male and oviducts in female amphibians.[2]

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[1] Burns, R. K., and Buyse, A., *J. Exp. Zool.*, 1934, 67.

In the preliminary experiments reported in this paper, newly metamorphosed and laboratory raised *Rana pipiens* were divided into 3 groups, one implanted with pituitaries, the second injected with estradiol, and the third constituting the controls. Both sexes were used. Whole pituitaries taken from adult preovulating female frogs were implanted subcutaneously 3 times weekly in the experimental animals. Each of the estradiol group of young frogs received .03 mg of estradiol, dissolved in sesame oil, per week, and the injections were intraperitoneal. The controls consisted of untreated, newly metamorphosed frogs, and some injected with sesame oil equivalent in amount to the experimental animals. Experimentals and controls were killed and the tissues fixed weekly up until 4 weeks after the initial treatment.

The gonads of the pituitary-treated animals showed marked growth, the testes becoming 5 to 10 times, and the ovaries 2 to 4 times the size of the corresponding controls. Histological observations demonstrated an increase of spermatogenesis in the treated males with the production of primary and secondary spermatocytes and some spermatids and the formation of well developed and enlarged seminiferous tubules. The tubules in the animals 1-2 weeks, after the initial treatment, were in most cases filled with the products of spermatogenesis, but those in the older animals were even more enlarged and practically empty, showing that the contents of the tubules had been forced out of the testes. No gross or microscopic effect was observed on the male Mullerian ducts.

The male controls showed slight seminiferous tubule formation, but these tubules were very small and in all cases were full of spermatogonia and some primary spermatocytes, but contained very few if any secondary spermatocytes and no spermatids. No mature spermatozoa were found in any of the experimentals or controls during the month following metamorphosis.

The ovaries of the pituitary-treated females were enlarged to 2 or 3 large lobes, while no more than one or 2 very small lobes were found in any of the controls. The oöcytes of these enlarged ovaries were slightly larger than those in the control ovaries. Both the experimental and control females had open oviducts, but these were minute, and the experimentals were only very slightly larger than controls.

Neither testes nor ovaries of the estradiol-treated animals showed any differences from the controls, but the estradiol-treated frogs showed a decided hypertrophy of the oviducts of the female and the Mullerian ducts of the male 8-10 days following initial injection. In all observed cases, both the Mullerian ducts and the oviducts were larger at the posterior ends. Sections of these ducts, 4 weeks after initial injection, showed large open tubules lined with columnar, granulated epithelium.

The controls, fixed at the same ages as the experimentals, showed very little growth, if any, of either the Mullerian ducts or oviducts. The Mullerian ducts of the 4-week-old control male and the oviducts of corresponding females were very thin and hardly visible strands. When sectioned, the Mullerian ducts appeared to very minute, closed circles of undifferentiated cells. Open oviducts were present in the female controls, but these were also extremely small and consisted of undifferentiated cells.

Summary. The ovaries and testes of recently metamorphosed frogs (*Rana pipiens*) responded by rapid growth and the testes also by cellular differentiation when the animals were treated with whole pituitary glands from mature females of the same species. Estradiol had no effect on the gonads but, in contrast with the pituitary hormone, estradiol brought about hypertrophy of both the oviducts and Mullerian ducts, causing the latter to simulate functional oviducts.

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[2] Foote, C. L., *Anat. Rec.*, 1939, **72**, suppl.; Puckett, W. O., *Anat. Rec.*, 1940, **75**, 127, suppl.; Eversole, W. J., and D'Angelo, S. A., *J. Exp. Zool.*, 1943, **92**, 215; Saunders, G. S., and Rugh, R., *J. Exp. Zool.*, 1944, **95**, 5; Glass, F. M., and Rugh, R., *J. Morph.*, 1944, **74**, 3; Adams, E. A., *Anat. Rec.*, 1945, **91**, 4, suppl.

15029

pH of the Chorio-allantoic Fluid of Chick Embryos Infected with Influenza Virus B.*

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Studies have been reported[1] on the rate of increase and the amounts of the influenza virus B (Lee strain) in the chorio-allantoic fluid of virus-infected chick embryos incubated at 35, 37, and 39°C. The increase in virus judged on the basis both of infectivity and hemagglutinative activity was greatest at 35°C and reached levels but little lower at 37°C. At 39°C the conditions were entirely unsuitable for the propagation of influenza virus B in chick embryos. While the temperature of incubation *per se* may influence the increase in influenza virus B in the chorio-allantoic fluid, it is possible that certain other factors, such as those concerned with the metabolism of the chick embryos, may be of great if not chief significance. Romanoff and Hayward[2] have shown that with incubation of normal chick embryos at 37.5°C, the pH of the chorio-allantoic fluid progressively changes from a reaction of high alkalinity, pH 8, on about the 10th day to a reaction of medium acidity, pH 5 to 6, on about the 14th day. The total period of incubation of the fertile egg at which these low pH levels are reached is dependent on the stage of development of the embryo, which in turn varies considerably with the temperature of incubation. In the studies[1] on the influence of the temperature of incubation on virus propagation, an investigation was made also of the pH changes occurring in the chorio-allantoic fluid of the same embryos. The findings revealed definite relations between the increase of the virus and the pH of the chorio-allantoic fluid which suggest a probable influence of pH on the amounts of virus demonstrable. The results of these studies on the relation of pH to the increase of influenza virus B (Lee strain) in the chorio-allantoic fluid of virus-infected embryos incubated for various intervals at 35, 37, and 39°C are described in the present paper.

Methods and Materials. The data were collected on the same embryos utilized in the work previously reported.[1] Eggs for the experiments were obtained from a commercial hatchery on the morning of the 11th day of incubation at 37.5°C. The eggs were inoculated into the chorio-allantoic sac at room temperature with 0.05 ml of a 10^{-3} dilution in hormone broth of stock virus. The openings ground in the shells for inoculation were sealed with paraffin, and the eggs were then incubated at the desired temperature which did not vary more than ± 0.25°C. The humidity was kept at a high level by means of open pans of water placed on the floor of the incubator.

At intervals the chorio-allantoic fluids of 10 embryos were drawn off individually into test tubes. The volumes of the fluids from

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[‡] Consultant to Secretary of War and Member, Commission on Acute Respiratory Diseases, Board for the Investigation and Control of Influenza and Other Epidemic Diseases in the Army, Preventive Medicine Service, Office of the Surgeon General, United States Army.

[1] McLean, I. W., Jr., Beard, D., Taylor, A. R., Sharp, D. G., Beard, J. W., Feller, A. E., and Dingle, J. H., *J. Immunol.*, 1944, **48**, 305.

[2] Romanoff, A. L., and Hayward, F. W., *Biol. Bull.*, 1943, **84**, 141.

TABLE I.
pH, Total Volume and Total Virus Yield (Red Blood Cell Agglutinative Activity) of Chorio-allantoic Fluid from Chick Embryos Incubated at 39, 37 and 35°C for Various Periods After Inoculation with Influenza Virus B (Lee Strain).

Time of Incubation Hr	Incubation Temp. °C	pH											Total volume, ml	Total virus, gamma
		Individual embryo fluids										Pooled aliquots		
		1	2	3	4	5	6	7	8	9	10			
6	39	6.7	6.5	7.9	7.2	8.2	7.3	6.6	7.4	8.3	6.5	7.3	66.9	0
	37	7.4	7.9	8.2	7.5	7.9	7.1	7.9	7.3	7.1	7.3	7.6	65.9	0
	35	—	—	—	—	—	—	—	—	—	—	7.4	66.9	0
12	39	6.5	6.7	7.6	7.1	7.8	7.8	7.5	7.8	7.9	6.4	7.5	64.5	0
	37	6.1	6.5	6.7	6.9	6.5	7.3	7.2	6.4	6.2	6.3	6.6	60.2	0
	35	—	—	—	—	—	—	—	—	—	—	7.4	57.9	0
18	39	6.5	6.7	6.7	6.5	6.6	6.9	7.2	6.8	7.1	7.0	6.8	63.0	0
	37	7.5	7.4	6.7	7.4	7.1	7.9	7.0	7.7	7.7	7.2	7.3	70.6	0
	35	—	—	—	—	—	—	—	—	—	—	7.5	51.9	16.5
24	39	7.2	6.6	7.2	7.3	7.0	7.5	6.9	7.4	6.2	6.5	7.4	63.6	0
	37	7.5	6.6	7.9	7.5	7.3	7.4	8.0	7.4	7.3	7.3	7.4	71.6	342
	35	—	—	—	—	—	—	—	—	—	—	7.6	60.3	1,450
36	39	6.3	6.9	6.3	7.3	6.5	6.0	6.9	6.7	6.6	6.3	6.7	65.5	21.5
	37	6.4	7.0	7.3	7.7	7.5	7.3	7.3	7.4	6.1	7.2	7.3	78.5	3,440
	35	—	—	—	—	—	—	—	—	—	—	7.7	56.5	4,390
48	39	6.6	6.5	7.0	6.5	7.6	6.3	5.8	6.6	6.1	6.5	6.7	59.1	51.4
	37	7.5	7.4	7.4	7.7	7.4	5.8	7.3	7.4	7.4	7.7	7.3	103.0	3,540
	35	—	—	—	—	—	—	—	—	—	—	7.7	76.7	5,380
60	39	6.6	6.0	5.8	5.7	6.2	6.4	6.5	6.3	6.7	6.5	6.4	53.6	0
	37	7.1	7.4	6.5	7.3	7.6	7.5	7.5	7.4	7.5	7.5	7.3	97.0	2,330
	35	—	—	—	—	—	—	—	—	—	—	7.9	86.0	5,920

the individual eggs were determined by weighing the tubes before and after the fluid was drawn into them. Aliquots of each group of 10 fluids were prepared by combining 1/10 of the volume of each respective fluid. Titrations were made of the hemagglutinative activity of each individual and each aliquot fluid for estimates of virus quantity. The method for converting the titration results, obtained in terms of titer, to the values representing absolute quantities of virus have been described.[1] Each aliquot fluid was titrated also for infectivity for chick embryos.

Measurements were made with the glass electrode of the pH of the individual fluids obtained after incubation of the eggs at 39 and 37°C and of the respective pooled aliquots of the fluids taken after incubation at 39, 37, and 35°C for the periods of 6, 12, 18, 24, 36, 48, and 60 hours of incubation. The individual fluids of the experiment at 35°C were not examined.

Results. The results are given in detail in Table I. The numbers assigned to the individual embryos in this table are the same as those given in Tables I (35°C), II (37°C), and III (39°C) previously reported[1] so that a direct comparison can be made between those results and the present findings with each individual embryo shown in Table I. A direct comparison of the findings with the aliquot fluids, likewise can be made for the same reason. In Fig. 1 are shown the trends of the changes in pH of the chorio-allantoic fluid with respect to the period of incubation at the various temperatures. For comparison there is shown also in Fig. 1 the curve representing the changes in the pH of the chorio-allantoic fluid of normal embryos incubated at 37.5°C as determined by Romanoff and Hayward.[2]

It will be noted in Table I that the pH of the individual fluids of the embryos incubated at 39°C showed a progressive decrease into the acid range after 24 hours' incubation. The pH of the aliquot fluid at 24 hours was 7.3, but the values for all of the aliquot fluids of the later periods were below pH 7.0, as shown in Fig. 1. The virus quantity indicated by hemagglutinative capacity was measurable only in the groups of embryos incubated for 36 and 48 hours. A low degree of virus infec-

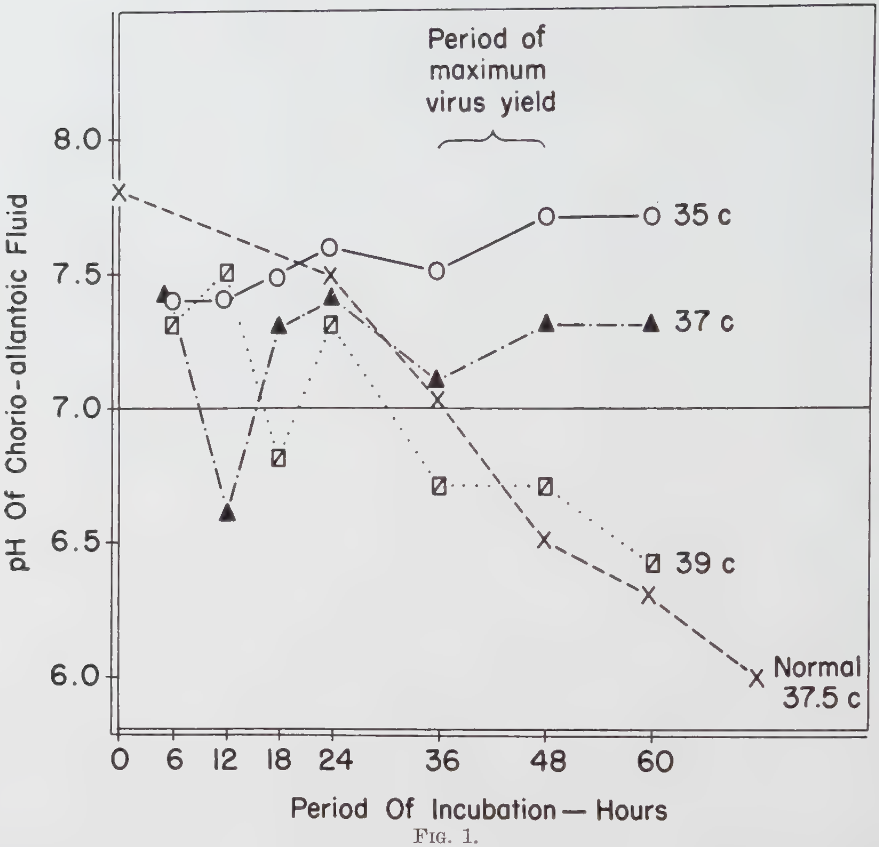

FIG. 1.

The relation of the pH of the chorio-allantoic fluid of embryos infected with influenza virus B (Lee strain) to the time of incubation at 35, 37, and 39°C after inoculation with the virus. The curve (broken line) characterized by the symbol (x) was constructed from the data of Romanoff and Hayward.[2] This curve indicates the changes which occur in the pH of the chorio-allantoic fluid during incubation at 37.5°C of normal chick embryos of the same stage of development as those infected with virus.

tivity for chick embryos was detectable[1] at all periods, reaching the highest value at 24 to 36 hours. The pH of the aliquot fluids from embryos incubated for 12 hours at 37°C was in the acid range as shown in Fig. 1. During the later periods of incubation, however, the pH was maintained above 7.0. The hemagglutinative capacity at this temperature of incubation was greatest at 36 to 48 hours, Table I, and was about 100 times that observed during the same incubation intervals at 39°C. The highest degree of virus infectivity observed[1] for these fluids was also reached at 36 to 48 hours and was likewise of the order of 100 times that observed for the corresponding groups incubated at 39°C. At the 35°C incubation temperature the pH of the aliquot fluids of the groups of 10 embryos showed no tendency to become acid. On the contrary, there was a tendency toward a general increase in alkalinity throughout the experiment. A steady increase in hemagglutinative capacity was observed throughout the 60-hour incubation period, and the greatest rate of increase occurred during the 18- to 36-hour period. The highest levels of infectivity,[1] which were reached at 36 and 48 hours, were 6 to 7 times higher than those observed at 37°C.

Discussion. The present observations reveal a striking relation between the pH of the chorio-allantoic fluid of chick embryos infected with influenza virus B (Lee strain) and the increase of the virus in the fluid. Though the significance of the relationship is not entirely clear, certain findings suggest that the pH of the chorio-allantoic fluid may be a principal factor limiting the increase or yield of the virus: (1) The pH range optimum for stability[3] of the influenza virus B is at or above 7.9, and, judging from loss of hemagglutinative capacity, the virus is rapidly inactivated and perhaps degraded even at 4°C, in the acid pH range. An acid reaction of the chorio-allantoic fluid is reached in 18-30 hours and maintained thereafter in embryos incubated at 39°C, while an alkaline reaction is maintained throughout in the fluids of embryos incubated at the lower temperatures. It is noteworthy that the definite acid reaction at 39°C is attained in the period before the occurrence of massive increase in virus. (2) In contrast with the behavior of influenza virus B, the influenza virus A (PR8 strain) reaches high titers[4] in the fluid of embryos incubated at 39°C. In further contrast is the observation that the pH range of optimum stability[3] of the influenza virus A is 6.5 to 7.0, which is nearly 2 pH increments below the optimum range for influenza virus B. The acid reaction developed in the fluid at 39°C would thus not be expected to influence greatly the increase of the influenza virus A. (3) The temperature differential between 37 and 39°C is relatively small with respect to direct influence on the virus in comparison with the pH differential, as shown in Fig. 1, which is great in view of the sensitivity of the influenza virus B to hydrogen ion concentration in this range. (4) There is evidence[5] that while influenza virus B as such is demonstrable only in small quantity by hemagglutinative and infectivity tests in the chorio-allantoic fluid of embryos incubated at 39°C, much may be formed only to be inactivated and partially broken down on exposure to the adverse pH of the chorio-allantoic fluid. This possibility has already been discussed[5] on the basis of sedimentation velocity and electron micrographic studies of material obtained from the fluid of embryos in which attempts were made to propagate influenza virus B at 39°C.

It is of interest to note the apparent influence of infection of the embryo with the virus on the hydrogen ion concentration of the chorio-allantoic fluids. This is especially well illustrated in the experiment in which incubation of the embryos was carried out at 37°C following inoculation with the virus. It is seen, Fig. 1, that an alkaline reaction was maintained throughout the later periods of the experiment, whereas the pH of the fluid of normal embryos of a comparable stage of development at 37.5°C becomes progressively more acid in similar periods. In the virus-infected embryos incubated at 35°C the pH of the chorio-allantoic fluid became progressively more alkaline. Only small effect was observed at 39°C. It appears likely that the establishment of massive infection of the embryo with virus leads to alteration of the processes of the excretion of acid metabolites with which is associated the change in the reaction of the chorio-allantoic fluid of normal embryos.

Summary. Influenza virus B (Lee strain) increases to small degree in the chorio-allantoic fluid of 11-day-old chick embryos incubated for 60 hours at 39°C after inoculation with the virus. Increase was great at 37°C and still greater at 35°C. The pH of the chorio-allantoic fluid of the same embryos descended into the acid region at 39°C; at 37°C the pH remained essentially above 7.0; and at 35°C the reaction was maintained well in the alkaline region. These relations suggest the possibility that the extent of increase of the virus in the chorio-allantoic fluid may be influenced more by the pH of the fluid at the various temperatures than by the temperature *per se.*

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15030 P

Clotting of Blood *In vivo* by Aqueous Homologous Lung Extract, Some Clinical Implications.*

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Pathological studies of the brain in patients dying of pneumonia encephalitis revealed primarily vascular thrombi and hemorrhages. The cerebral lesions were similar regardless of the etiological agent causing the pneumonia, thus suggesting that the cerebral lesions were probably produced by some product of the injured lung tissue.

In an attempt to produce experimentally the lesions of pneumonia encephalitis, rabbit lungs were homogenized with a tissue homogenizer which reduced the tissues to the consistency of a gruel. This homogenized tissue was then diluted 1 to 25 with saline solution and filtered through paper. When this saline extract was injected intravenously into rabbits in amounts of .5 to 1 cc, death ensued in from 30 seconds to 2 minutes. This rabbit lung extract proved to be rapidly fatal to all animals of the species producing nystagmus, ataxia, convulsions, and death. Intravenous injections of similar or larger amounts of extracts of other organs such as brain, liver, kidney, intestine, spleen, and nasal mucosa produced no untoward effect. In order to determine whether or not the toxic action of lung was species specific, rabbits were inoculated intravenously with a 1 to 25 aqueous extract of beef, pork, sheep, guinea pig, and human lung. Of these heterologous lung extracts only the beef lung proved toxic to rabbits, giving a delayed reaction with subsequent death of the animal. Additional proof of the species specificity of the lung extract was demonstrated by intravenous inoculation into guinea pigs and dogs of an aqueous extract of the homologous lung tissue. In each case the inoculated material caused rapid death of the animals. From these experiments it is apparent that lung tissue extract when injected intravenously is fatal to the homologous species. Subcutaneous and intraperitoneal injections even in massive doses were innocuous.

Animals sacrificed after receiving repeated sublethal intravenous doses of lung extract and finally killed with a lethal dose, revealed within the cerebral tissues lesions resembling those seen in human cases of pneumonia encephalitis. The vessels showed early thrombosis with a deposition of fibrin clot. Further proof that the observed reaction was due to clotting is supported by the observation that all symptoms were prevented by preliminary intravenous injection of 10 mg of heparin.

We were intrigued by the fact that lung extracts from animals of other species were innocuous even when given in much larger doses than the homologous lung tissue. Extracts of lung tissue, whether from the rabbit or guinea pig, cause blood to gel in a test tube almost instantly, regardless of the animal species from which the blood is derived. The contractility of these clots is rapid and extensive, often contracting to one-half of the original volume of the clots within 2 or 3 hours. It is, therefore, apparent that some factors are operative in the animal body which prevent clotting, which are not present in the test tube experiment. Rabbits which had received an intravenous injection of guinea pig lung extract were found to be protected when given intravenous doses of homologous lung tissue extract, provided an interval of about 10 minutes elapsed between the two injections. When the extracts of guinea pig and rabbit lungs were mixed and given simultaneously, death ensued promptly. It is, therefore, apparent that the guinea pig lung extract sets up a protective mechanism against the homologous lung extract within a period of 10 minutes. The same mechanism may be mobilized by the injections of fresh human and guinea pig sera. The protection is transient, lasting more than one hour but less than 4 hours. Inactivated human and guinea pig sera afford no protection. Whether the protective action is due to the complement in the fresh sera, or whether other thermolabile enzymes are responsible for the reaction, has as yet not been determined. We are not prepared at this time to identify the substance in the aqueous extract of lung tissue which initiates the clotting. Lung tissue extract which has been heated at 56°C for 30 minutes is inactive. This would suggest that either pro-thrombin or thrombin may be the substances responsible for the clotting since they are thermolabile, while kinases as a class are thermostabile. Further studies are now under way which, it is hoped, will throw light on this subject.

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15031

Effects of Thiouracil on the Mammary Gland.*

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The thyroid-inhibiting substances, thiourea and thiouracil, induced hyperplasia of the thyroid, attended by typical symptoms of hypothyroidism as indicated by lowered metabolism, decreased food intake and retarded growth.[1-5] In treated animals no other tissues except the anterior lobe of the pituitary were reported altered histologically.[1,5] The weights of the pituitary, gonads, and adrenals of treated animals were in proportion to the decreased body weights. The physiological effects of the thyroid-inhibiting substances were nullified by giving thyroid hormone simultaneously.[2,3]

Since thyroidectomy in both male[6] and female[7] rats favored the stimulation of lobule-alveolar development and the inhibition of the extension of the ducts of the mammary glands, an investigation was made to compare the effects of thiouracil with those following thyroidectomy on the mammary structure of the rat.

Methods. Rats of the Long-Evans strain were arranged in groups of similar age and body weight, and as far as possible comparisons were made between animals of the same litter. Eighty-five rats of both sexes, with body weights ranging from 37-238 g, were used. In some experiments rats of both sexes were castrated before treatment and a few animals were thyroidectomized for additional controls. Since previous experience had shown that injections of estrogen magnified the effects of thyroidectomy on mammary growth,[6] several experiments were made in which total dosages of 50 γ of a-estradiol dipropionate† were injected during the last 10 days of the experimental period. In one group, thyroxin was administered at the rate of 5 γ daily to determine if the effects of thiouracil would be counteracted. This dose of thyroxin was found to nullify the effect of the doses of thiouracil given on the metabolic rate and thyroid

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* Aided by a grant from the Sage Fund, of the Trustee-Faculty Committee on Research.

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† The estrogen alpha-estradiol dipropionate (Di-Ovocylin) was obtained through the courtesy of Dr. E. Oppenheimer of the Ciba Pharmaceutical Co., Summit, N.J.

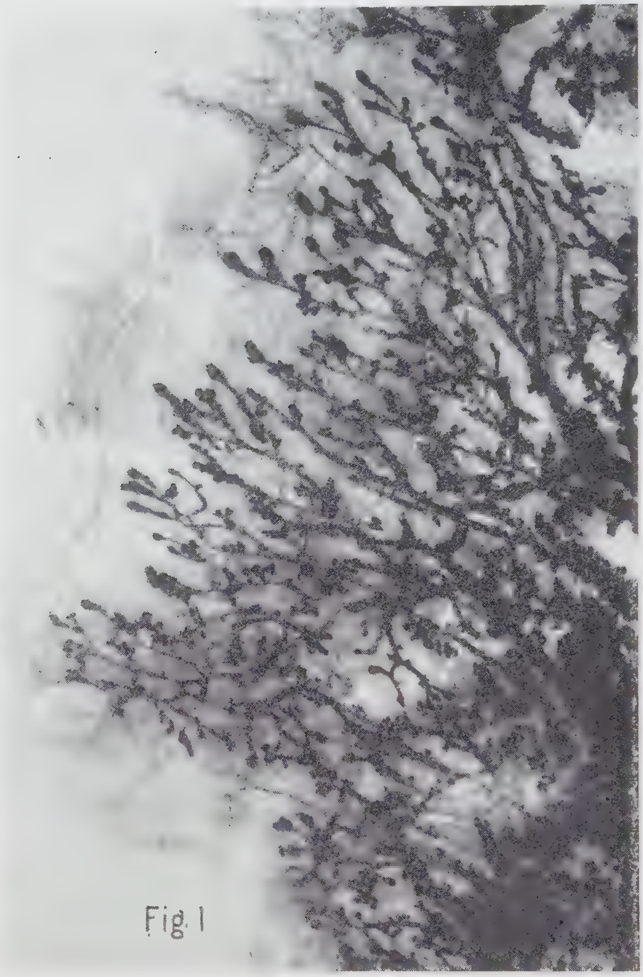

Fig. 1

Mammary gland of young castrated female rat which received 5 γ of alpha-estradiol dipropionate daily for 10 days.

hyperplasia.[8,9]

Thiouracil[‡] was administered subcutaneously in an aqueous suspension in daily doses of 10 mg per 100 g body weight or in the drinking water as a 0.1% solution. Treatment was continued for periods of 18-35 days. At autopsy, the right abdominal mammary gland was removed and stained as a whole mount. Observations were made on the thyroids, adrenals, and body weights.

Results. The mammary glands of 30 normal male and female rats treated with thiouracil for 18-35 days were indistinguishable from glands of 13 control animals. In a few instances there was observed a slight thickening of the ducts and an inhibition of the duct extension in the treated rats. The thyroids were increased 3 to 4 times in weight and the adrenals weighed less those of the controls but were in proportion to the body weight. The treated animals failed to grow as well as the controls. The estrous cycle was lengthened to 7-9 days in the adult treated females.

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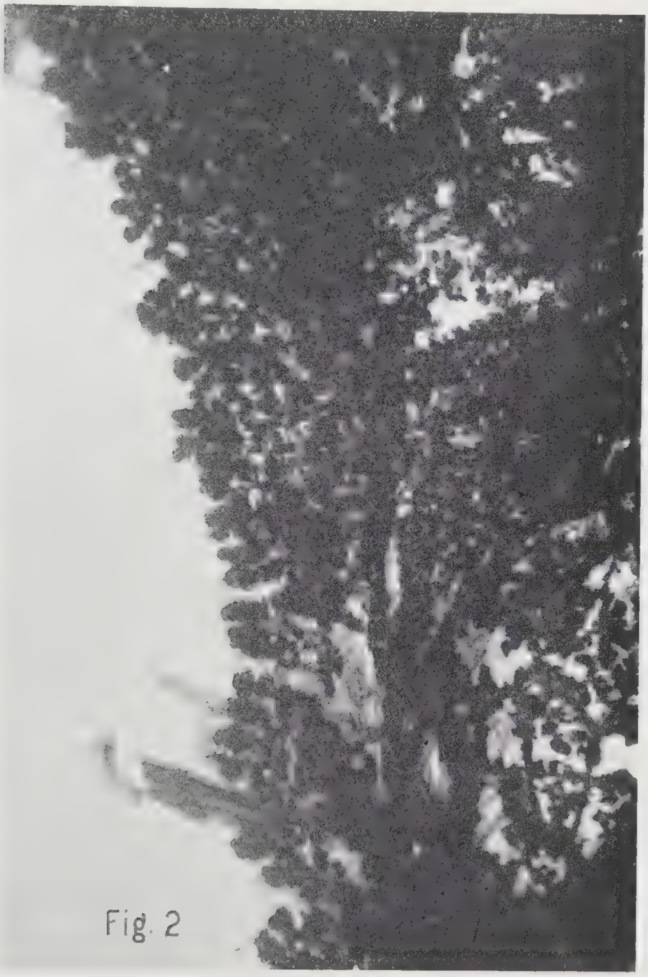

Fig. 2

Mammary gland of castrated female littermate which received thiouracil for 24 days, and the same amount of estrogen during the last 10 days.

Comparison of the glands of 4 castrated treated rats with those of the 4 controls likewise indicated little or no differences in mammary structure.

When estrogen was injected in the thiouracil-treated and control rats, the mammary glands were obviously different. The glands from 13 rats receiving thiouracil possessed well developed lobules and alveoli while those from the 8 controls showed the usual estrogen stimulation on growth of the end buds and extension of the duct system (compare Fig. 1 and 2). When thyroxin was administered at the level of 5 γ per day, in addition to the thiouracil and estrogen (7 rats), the mammary glands showed end bud growth and duct extension with only an occasional lobule. The extensive lobule-alveolar growth failed to appear in these glands. The thyroids of these rats failed to show the great degree of hyperplasia that occurred when thiouracil alone was given.

Discussion. Thiouracil was unable to produce the lobule-alveolar growth in the mammary glands of normal or castrated rats similar to that produced by thyroidectomy.[6,7] This difference cannot be explained by a failure of the drug to induce a hypothyroid state because when estrogen was administered the lobule-alveolar development appeared as it did in thyroidectomized rats similarly treated. It is possible that the period of thiouracil treatment was not continued for sufficient time for the changes to occur although thyroid removal for a similar period (35 days) did bring about these changes.[7] A lengthened estrous cycle was also produced by thiouracil, similar to that found in thyroidectomized rats.[10]

Estrogen injections into castrated rats will in time produce lobule-alveolar development but not in the 10-day periods of these experiments.[11] The hypothyroid state apparently causes a precocious appearance of the lobules and alveoli in the mammary glands under these conditions. The injections of thyroxin to offset the effects of thiouracil in inducing hypothyroidism were manifested in the failure of lobule-alveolar growth to appear in the mammary glands. Whether hypothyroidism brings about these changes in the mammary glands by altered hormone metabolism or by altered tissue responses is still a matter of conjecture.

Conclusion. Thiouracil administered to normal or castrated male and female rats failed to induce an alteration of the mammary glands similar to that obtained following thyroidectomy. The mammary glands of rats treated with thiouracil and with estrogen for the last 10 days of the experimental period possessed well developed lobules and alveoli whereas the control glands showed end bud growth and duct extension. When thyroxin was given along with thiouracil and estrogen, the mammary glands resembled those from rats receiving estrogen alone and no precocious lobule-alveolar growth was noted.

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15032

The Use of Regenerating Liver as a Method of Assay.*

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Although the rate of mitosis in regenerating liver has been studied,[1] the procedure used was so laborious and time-consuming as to preclude its use as a method for testing the effect of agents upon the growth of liver cells. By applying the technic for obtaining free nuclei previously described by one of us,[2] a method of assay has been developed by means of which it has been possible to test as many as 10 substances in a week. The results of these assays will be published elsewhere.[3] We wish here to present results of studies of the relation of the age of the animal to the rate of mitosis and the uptake of P^{32} by the regenerating liver.

Method. An incision was made on the midline extending about ¼ to ½ inch on either side of the xiphoid process in an antero-posterior direction. The abdominal wall was cut along the midline for ¼ inch posterior to the tip of the xiphoid process which was dissected free of its attached muscle and fascia. With gentle pressure on the sides of the animal the median and left lateral lobes were delivered to the surface and their fascial attachments cut. They were then tied off at the base with cotton or linen thread and cut off as close as possible to the tie. To minimize

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* The work described in this paper was done under a contract recommended by the Committee on Medical Research between the Office of Scientific Research and Development and the University of California.

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TABLE I.
Liver 3 Hours After Intravenous P^{32}.

	% mitosis						P^{32}/g liver as % injected dose		
Day post-op.	n	1 mo	n	4-6 mo	n	16 mo	1 mo	4-6 mo	16 mo
0 *s* hep.	5	.07	6	.00	4	.01	3.16	1.78	1.78
0 *c* hep	3	.07	6	.03	4	.07	6.08	2.77	2.69
1	17	.74	8	.37	4	.25	5.52	2.81	2.19
2	5	.23	7	.47	4	.51	5.52	2.41	2.40
3	4	.20	6	.27	4	.98	4.86	2.25	2.43
4	3	.12	2	.18	4	.39	4.86	1.87	2.25
5	3	.06	4	.05	4	.13	3.51	1.88	2.14

n = No. of animals.

TABLE II.
Liver 24 Hours After Subcutaneous P^{32}.

	Days post-operative					
	1	2	3	4	5	6
% P^{32}/g liver	2.18	2.11	1.87	1.77	1.90	1.93
m	.047	.066	.054	.100	.115	.095
n	15	10	12	6	6	5
% Mitosis	.73	.28	.21	.07	.08	—

m = standard error of the mean.
n = No. of animals.

infection aseptic precautions were used throughout the operation. On a diet of Purina dog chow survival was 100% unless there was a gross error in the technic, *e.g.*, perforation of the diaphragm or cutting a large blood vessel. When the animals were put on a prepared diet containing 10 to 13% fat before the operation, survival was only 30 to 40%.

Twenty-four hours after the partial hepatectomy the animals were given P^{32} as Na_2HPO_4 intravenously via the femoral vein in doses not exceeding 1 mg Na_2HPO_4, 1.5 μc P^{32}/g of body weight. Three hours later the liver was perfused and removed for assay. In another set of experiments the P^{32} was given subcutaneously immediately after the operation and the liver was removed 27 hours later. A small portion of the liver was accurately weighed and its P^{32} content determined with a Geiger-Muller counter. For simplicity only nuclei in metaphase and anaphase were counted as being in mitosis. In all cases, the liver was removed between 1:30 and 4:00 p.m.

Rats of a hooded strain inbred by brother-sister mating in this laboratory for 10 generations were used in these experiments. Purina dog chow was fed *ad libitum*. The animals 1 month old weighed from 45 to 55 g, those from 4 to 6 months old weighed from 170 to 215 g, and those 16 months old weighed from 240 to 320 g. The animals in each group were given the same dose per gram of body weight on the basis of the average weight for each group, 50, 200, and 250 g respectively. No attempt was made to adjust the dose administered to the body weight within any one group. In calculating the percentage of P^{32} per gram of liver, the data were corrected to the average weight of the group, *e.g.*, to 50 g in the first group by multiplying by the observed weight and dividing by 50. From experiments to be reported subsequently, data are available for testing the validity of this correction. In 19 experiments, including 84 rats 1 month old, the weight ranged from 30 to 60 g, with a large majority between 45 and 55 g, and the mean percentage uptake of P^{32} 27 hours after operation and 4 hours after the injection of P^{32} was $4.517 \pm .084$ for the corrected and $4.728 \pm .120$ for the uncorrected. The variance ratio was 2.01 and $P = 0.1 - 1.0\%$, that is, the variance is significantly greater in the uncorrected data.

The regression coefficients of the percentage of P^{32} on weight and their standard errors were calculated for the first 15 experiments (61 rats), since in the last 4 experiments there was no significant variation in weight. These were -0.09 ± 0.02 before and -0.008 ± 0.02 after correction, the effect of weight thus being almost entirely eliminated by the correction.

In Table I are shown the percentage of nuclei in mitosis and the P^{32} per gram of perfused liver at from 4 hours to 5 days after partial hepatectomy. Mitosis reached a maximum on the first, second, and third days for animals 1, 5, and 16 months old respectively. P^{32} was at a maximum at 0, 1, and 2 days for the same groups. There was an apparent correlation between the time required for reaching maximum mitosis and that for reaching the maximum uptake of P^{32}, the latter anticipating mitosis by 1 day. There was no significant change in mitotic rate during the first 4 hours after the operation.

In another set of experiments with rats weighing 50 g the P^{32} was given subcutaneously 24 hours before the liver was removed for assay. These results are shown in Table II. Maximum mitosis and uptake of P^{32} were reached at 1 day. Whether the P^{32} was administered intravenously or subcutaneously, the uptake of the radioactive element was a much less sensitive index of mitotic function of the liver than was the mitotic count itself. For example, while the uptake of P^{32} varied only a few percent between 0 and 1 a day, the mitotic rate increased by more than 1000%.

In animals which received P^{32} intravenously 1 hour after the partial hepatectomy, a greater concentration of P^{32} was found in the liver than in non-hepatectomized animals, which is probably due simply to the decrease in liver volume. This suggests that the progressive decrease of P^{32} found in the liver between the first and fifth days may be more directly related to the increase in bulk of the liver rather than changes in mitotic activity. However, the data cannot be considered to prove that P^{32} uptake is determined exclusively either by changes in liver volume or rate of mitosis.

Conclusions. 1. The time at which mitosis in regenerating liver reaches a maximum varies with the age of the animal. 2. P^{32} uptake by regenerating liver, 3 hours after intravenous administration of labelled Na_2HPO_4, varies with time after partial hepatectomy. This variation may be related to changes in liver volume or to mitotic activity or both. 3. P^{32} retention 27 hours after subcutaneous administration of labelled Na_2HPO_4, shows little variation with time after operation. 4. The mitotic rate provides a useful index which may be used in assaying the effect of various agents on the reproduction of liver cells.

15033

On the Use of Sodium Sulfadiazine in Surgery on Amphibian Embryos.

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One of the greatest difficulties encountered in surgery on amphibian embryos (especially Amblystoma) has been the unpredictable mortality. Particularly in experiments upon the central nervous system, if aseptic procedures are not followed, the death rate may be discouragingly high. Long experience has shown that complete superficial healing, following surgical interruption of the central nervous system, is no guarantee of survival. Embryos, in which the wound surfaces are com-

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* Guest Investigator. Medical Officer of the Atlantic Division of Pan American Airways, Inc.

TABLE I. Showing Survival of Operated Amblystoma Embryos When Treated with a 1% Solution of Sodium Sulfadiazine as Compared with Those Kept in Spring Water. The Right Brachial Region of the Spinal Cord Was Removed (in 0.4% NaCl) from Embryos in Stages 20-24 (wound not covered).

Group	Egg clutch	No. of operations	Stage	Time (hr) after operation when transferred to: Sodium sulfadiazine	Spring water	No.	Survival (days after operation): 1	2	3	4	5	6	7	8	Total No.	% survival
A	A	20	20	a 10		10	10	10	10	10	10	10	10	10	10	100
				b	10	10	10	10	3	1	1	1	1	1	1	10
B	B	20	21+	a 3		10	10	10	10	10	10	10	10	10	10	100
				b	3	10	10	10	1	0	0	0	0	0	0	0
C	C	70	22—	a 2		35	35	35	35	35	35	34	34	34	34	97.1
				b	2	35	35	32	0	0	0	0	0	0	0	0
D	D	30	21	a 8		15	15	15	15	15	15	13	12	12	12	80
				b	8	15	15	9	0	0	0	0	0	0	0	0
R	—	50	23-24	a 12		25	25	25	25	25	25	25	24	24	24	96
				b	12	25	25	25	20	12	0	0	0	0	0	0

Group A—All embryos in Aa transferred to spring water on third day after operation.
Group B—All embryos in Ba retained in sodium sulfadiazine for 8 days.
Group C—18 embryos in Ca transferred to spring water on second day. Remainder kept in sodium sulfadiazine for 8 days.
Group D—7 embryos in Da transferred to spring water on third day. Remainder kept in sodium sulfadiazine for 8 days.
Group R—All embryos in Ra kept in sodium sulfadiazine for 8 days.

TABLE II. Showing Delayed Healing of Wounds in Operated Embryos Placed in 1% Solution Sodium Sulfadiazine (Operation described in Table I).

Group	Days after operation	Sodium sulfadiazine: No. of embryos	Healed	Almost healed	Slightly healed	Living	Spring water: No. of embryos	Healed	Almost healed	Slightly healed	Dead
A	2	10	0	2	8		10	4	2	4	0
	3	10	1	4	5		4		2	2	6
	4	10	10	0	0	10	1			1	9
B	2	10	0	7	3		10	0	10	0	
	3	10	5	5	0		1	0	0	1	9
	4	10	10	0	0	10	0				10
C	1	35	0	0	35		35	5	18	12	0
	2	35	0	19	16		32	12	9	11	3
	3 a	17	12	5	0						
	b	18	17	1	0		0				35
	4	35	35	0	0	35	0				35
D	1	15	0	8	7		15	2	13	0	
	2	15	0	6	9		12	10	2		3
	3	15	1	10	4		0				15
	4	15	14	1	0	15	0				15

A—Embryos placed in sodium sulfadiazine 10 hr after operation. Changed to spring water 3 days after operation.
B—Embryos placed in sodium sulfadiazine 3 hr after operation. Retained in sodium sulfadiazine.
C—Embryos placed in sodium sulfadiazine 2 hr after operation. Ca retained in sodium sulfadiazine. Cb transferred to spring water 2 days after operation.
D—Embryos placed in sodium sulfadiazine 8 hr after operation. Retained in sodium sulfadiazine.

pletely closed, frequently break down in regions remote from the site of operation. When disintegration of the embryo takes place it usually occurs 3 to 5 days after the operation.

Experience has shown also that survival varies with different clutches of eggs. Some eggs are presumably more hardy than others. The same type of operation, under identical technical procedures, may (with one egg mass) result in low mortality; in another it may be high. Evidence from long term experience indicates too that viability may vary with eggs from different geographical localities and from year to year.

In 1930, Woerdeman[1] described a method of sterile technic which insured a high percentage of survival in urodele embryos as compared with non-sterile procedures. This method with modifications has been employed by experimental embryologists (Harrison, Schotté, Detwiler, and others) with considerable success in recent years. It involves sterilization of all glassware, operating fluids, and operating instruments. The embryos are transferred successively through 9 to 10 washings in sterile water—using a sterile pipette for each transfer. Whereas this procedure, on the whole, has greatly lowered the mortality, the method cannot be regarded as entirely aseptic in the bacteriological sense, for the embryos are merely washed repeatedly and are probably not rendered entirely free from bacterial invasion into open wounds. Personal experience (Detwiler) over a period of 8 years has shown that this technic, although distinctively advantageous, does not always guarantee the expected results. Usually, however, the markedly greater survival has justified its use despite its laboriousness.

During the spring operating season (1945) we attempted to study restitution of the spinal cord, and subsequent limb function following unilateral excision of the brachial region.[†] In embryos with uncovered wounds, we encountered almost 100% mortality. Only 3 of 280 operated embryos survived. As a result of this discouraging yield we ventured to study the possibility of employing sulfa drugs. The results have not only been gratifying, but far beyond expectations. After preliminary tests, it was found that young embryos would undergo normal development and growth when kept in a 1% solution of sodium sulfadiazine (Sharp and Dohme) made up in spring water (1000 mg per 100 cc).

The operations reported above, and which resulted in nearly 100% mortality, were repeated. Following the operation, which was done in 0.4 NaCl solution, the embryos were divided into two equal groups. The specimens of one group were placed in individual Syracuse dishes containing a 1% solution of sodium sulfadiazine; those of the other group were placed in individual dishes containing spring water. The intervals in hours between the time of operation and transfer of the embryos to either sodium sulfadiazine or spring water are stated in Table I, which gives the results of 5 series involving 190 embryos.

In order to eliminate any possible modifying factor due to egg selection, records were kept on embryos from individual clutches in 4 of the 5 series (A, B, C, D). Of the 90 embryos kept in spring water, only 1 survived the fourth day, whereas of those treated with sodium sulfadiazine, only 5 out of 95 succumbed and 2 of these were 'unhealthy' appearing embryos when the operation was performed. Embryos transferred from the sodium sulfadiazine solution to spring water 2 to 3 days after the operation survived as well as did those maintained for longer periods in the solution of the drug (Table I). Whereas Table I and Fig. 1 give the survivals for 8 days only, it may be pointed out that the larvæ continued to survive until such time as they were discarded because of curvature or were fixed for microscopic study. Complete data on survival are given in Table I. The significant data are shown graphically in Fig. 1. Observations on the healing of the wounds show that, although drug treatment resulted in a 95% survival in these experiments, it did delay healing. The data are given in Table II. Here it is seen that none of the 70 embryos in the sodium sulfadiazine solution were healed on the second day after

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[1] Woerdeman, M. W., *Roux's Arch.*, 1930, **121**, 524.

[†] The operations were done without sterile precautions, and mostly upon embryos just after complete closure of the neural folds.

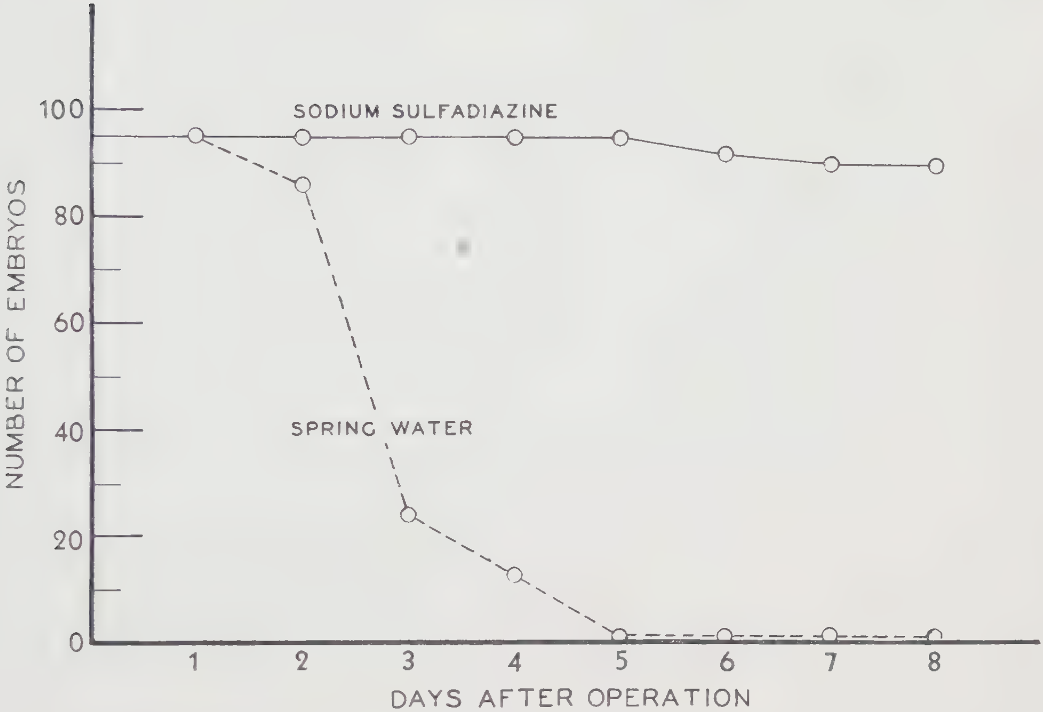

FIG. 1.

A summary of data listed in Table I. The solid line represents survival of embryos transferred after operation to a 1% solution of sodium sulfadiazine; the broken line gives the survival of those kept in spring water.

the operation, whereas 26 of the 70 embryos in spring water had healed over completely. Despite this, however, all but 5 of the embryos in spring water were dead on the third day, whereas 36 of the 70 treated embryos were healed. On the fourth day all sulfa-treated embryos but one were completely healed. It is apparent that death following complete superficial wound healing is due to internal infection and subsequent breakdown. This may be regional involving most frequently the head, or it may involve the whole embryo, with general disintegration.

In one series of unilateral brachial cord excisions in which the wound was covered with ectoderm and underlying mesoderm, the mortality rate was low. Out of 20 embryos kept in spring water, only 5 died (25%). In another group of 20 which were kept in sodium sulfadiazine for 3 days, all embryos survived. Covering the wounds, following extirpation of a portion of the central nervous system, usually insures a higher percentage of survival than in those with uncovered wounds. But even in embryos with covered wounds, the death rate may be high if sterile procedures are not employed, and if the material is not hardy.

The therapeutic value of sodium sulfadiazine is strikingly illustrated by the results obtained by Dr. Ging-Hsi Wang.[‡] He made grafts between the medulla and the brachial cord and vice versa (without sterile technical procedure), and obtained only 10 survivals out of nearly 300 operations. After the beneficial effects of the drug were discovered, he repeated the same type of operation upon 24 embryos and obtained 21 survivals. All the more striking are these latter results since the operations were done on embryos which had been kept in the refrigerator for nearly 4 weeks at a temperature of 5°C. Such material typically yields poor results.

[‡] Director, Institute of Psychology, Academia Sinica, Chungking, China. Guest investigator under the auspices of the Division of Cultural Cooperation, Department of State, U. S. Government.

Although we have not determined the minimum concentration and length of time necessary to insure survival with the sodium sulfadiazine treatment after operation, our results do show that treatment with a 1% solution for 2 days is adequate (Table I, Group Ca). Furthermore, operated embryos treated for 8-10 days show no deleterious effects of the drug. The developmental rate keeps pace with the controls provided the temperature is the same for both groups. The concentration which we used probably is much stronger than necessary to insure maximum survival. Unfortunately our observations were not made until near the termination of the operating season, and adequate material was not available this year to make further projected studies.

Observations show that even prolonged treatment with the drug has no visible effect upon growth and normal physiological function. This is apparent from a study of two series as follows: Normal embryos in stage 21 were placed in the sodium sulfadiazine solution and maintained there for 4 weeks. Since sulfadiazine gradually crystallized out in dishes containing the embryo, the solution was augmented several times during the first 2 weeks by the addition of freshly made stock solution. During the last 2 weeks, the solution became diluted gradually because of the daily addition of daphnia introduced with a pipette. Throughout this period the animals showed no deviations in development and behavior from those kept in spring water.

In one series (Group 1), 6 embryos were placed in sodium sulfadiazine solution and 6 in spring water. Fifteen days later (just prior to the feeding stage) the 6 treated larvæ averaged 15.8 mm in length; the 6 in spring water averaged 15.3 mm. In another series (Group 2), after 14 days, 6 treated larvæ averaged 14.8 mm, and 6 control embryos averaged 14.5 mm in length. All of these larvæ, whether treated or not, began feeding at the same stage (stage 45-46). At 21 days (7 days after the feeding stage) the treated larvæ in Group 1 averaged 19.3 mm in length; those in spring water averaged 18.6 mm. In Group 2 (6 days after feeding) the average length for both treated and control larvæ was the same (17.6 mm). At the end of 4 weeks, under similar conditions of feeding and temperature, the lengths of the sulfa-treated larvæ in Group 1 averaged 21.6 mm; the spring water controls averaged 22.4 mm. The treated larvæ in Group 2 averaged 22.1 mm; those in spring water averaged 22.1 mm.

Although there are many aspects of the problem which call for further work, nevertheless the present experiments indicate clearly the beneficial use of this drug in surgical procedures on the amphibian embryo.

Summary. The therapeutic effects of sodium sulfadiazine upon Amblystoma embryos, following surgical interruption of the central nervous system, are described. Embryos which may undergo 100% mortality when sterile technic is not employed, will survive when treated for 2 days with a 1% aqueous solution of the drug. There is no evidence of any toxic effect. Treated embryos undergo normal development and at a rate parallel with control embryos kept at the same temperature.

Minimum concentrations of the drug as well as minimum length of treatment necessary to insure survival remain to be determined.

15034

Some Factors Affecting Duration of Life in Total Anuria.*

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In a series of studies planned to inquire into the pathological physiology of the anurias, we first attempted to determine the factors which might allow the longest life expectancy in the experimental animal after the induction of total anuria. Results obtained on 61 dogs following total nephrectomy are discussed in this report.

Three to 7 days appears to be the usual duration of life in dogs rendered totally anuric by binephrectomy.[1,2,3] Some animals have been kept alive for longer periods under special conditions.[3] Obstruction of the renal arteries or of the ureters[4,5,6,7] frequently leads not only to anuria but also to acute nephrogenic hypertension. Total obstruction of the renal veins often is followed by massive hemorrhage into the renal capsule with death sometimes occurring in a few hours.[8] Obstruction of the renal arteries and veins is equivalent to removal of both kidneys, but this method leaves a mass of necrotic tissue *in situ*. In the present study, binephrectomy was the method of choice to produce anuria. The kidneys were approached by lumbar incision and removed without entering the peritoneum. Ether or nembutal anesthesia was used.

The binephrectomized dog appears outwardly normal for 24 to 48 hours postoperatively. On the second or third day he loses his interest in food but usually begins to show a marked thirst. At this time the animal will drink large quantities of water, quickly vomit, and then repeat this cycle over and over again. If this process is not prevented by limiting the water intake, loss of chlorides will lead to hypochloremia and early death. Vomiting can usually be controlled by giving water in small amounts (25 cc) several times daily.

Results. Binephrectomy in a single operation. The post-operative duration of life ranged from 60 to 130 hours, averaging 85 hours in a series of 16 dogs subjected to binephrectomy in a single operation. The data on this group of animals and those subsequently discussed are given in Table I.

Binephrectomy in two stages. To reduce the trauma experienced by the animals at the

TABLE I.
Hours of Life After Bi-nephrectomy.

One-stage operation		Two-stage operation		
No DCA	DCA treated	No DCA	DCA treated	DCA pre-treated
60	76	58	110	132
60	108	60	115	165
60	110	72	123	170
65	110	92	196	206
72	144	100	223	230
73	145	110		235
77	146	115		
78	148	115		
80	168	120		
84	173	120		
85	185	130		
85	200	130		
97		130		
127		140		
130		178		
130		192		
Avg 85	143	116	153	190
Stand. Dev. ±23	±34.5	±35.4	±47	±37

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* Aided by the A. D. Nast Fund for Cardiovascular Research. The Department is supported in part by the Michael Reese Research Foundation.

† Serving as a First Lieutenant in the Army Air Forces.

[1] Hoff, H. E., Smith, P. K., and Winkler, A. W., *J. Clin. Invest.*, 1941, **20**, 607.

[2] Kohlstaedt, K. G., Page, I. H., and Helmer, O. M., *Am. Heart J.*, 1940, **19**, 92.

[3] Winternitz, M. C., Mylon, E., Waters, L. L., and Katzenstein, R., *Yale J. Biol. and Med.*, 1940, **12**, 623.

[4] Hartwich, A., *Ztschr. f. d. ges. exp. med.*, 1930, **69**, 462.

[5] Katz, L. N., Landowne, M., and Friedberg, L., *Cardiologia,* 1941, **5**, 25.

[6] Megibow, R. S., Katz, L. N., and Rodbard, S., *Am. J. Med. Sci.*, 1942, **204**, 340.

[7] Taquini, A. C., *Am. Heart J.*, 1940, **19**, 513.

[8] Friedberg, L., *Am. Heart J.*, 1944, **28**, 786.

time of the induction of the anuric state, 16 dogs were subjected to binephrectomy in two stages: uninephrectomy was performed 1 to 4 weeks before the remaining kidney was removed. After removal of the second kidney, these animals survived from 58 to 192 hours, averaging 116 hours.

These results suggest that the reduced trauma, due to the need for only unilateral surgery at the time of induction of the anuria, allowed a significant increase in the duration of life.

Binephrectomy in one operation and post-operative treatment with DCA. Reports that desoxycorticosterone acetate (DCA) increased the life expectancy in certain conditions associated with trauma[9,10] suggested that this substance might act in a similar fashion to reduce the apparent effect of trauma in our animals. A group of 12 dogs subjected to binephrectomy in one operation was given subcutaneously 10 to 50 mg[‡] of DCA post-operatively and at intervals of 24 hours thereafter. These animals survived from 76 to 200 hours, averaging 143 hours.

These results suggested that the DCA favorably affected the survival time in these animals.

Binephrectomy in two stages and post-operative treatment with DCA. The above results suggested that perhaps a combination of reduced trauma attendant upon the two-stage operation, together with the use of DCA, might further increase the duration of life of the totally anuric animal.

A group of 5 dogs was uninephrectomized 2 weeks before removal of the second kidney. Twenty mg of DCA was administered subcutaneously post-operatively and at intervals of 24 hours thereafter. These animals survived from 110 to 223 hours, averaging 153 hours.

Binephrectomy in two stages with DCA pre-treatment. Previous reports suggested that a contributing cause of death in anuric animals surviving more than 3 days might be the accumulation of potassium in the plasma until its concentration was toxic for the heart.[11] Chemical and electrocardiographic studies on our dogs confirmed this finding. The plasma potassium level in the normal dog, using a modified chloroplatinate method, averaged 18 mg %. After binephrectomy this increased at about 4 mg % per day until the plasma level was about 40 to 50 mg %, a concentration which causes increased irritability of the heart and may lead to ventricular fibrillation.[1]

It was felt that the duration of life after total nephrectomy might be further increased by the use of DCA pre-operatively, since it has been shown[12] that DCA given to normal dogs causes an increased excretion of potassium, and when continued for a period of 10 days may lower the plasma potassium to about half its normal level. Starting with a lowered initial level of plasma potassium, it was thought that a longer survival in anuria might occur before a toxic concentration of plasma potassium would be reached.

Six uninephrectomized dogs were given 20 mg of DCA intramuscularly daily for a period of 10 days, with lowering of the plasma potassium from 18 mg % to an average of 11 mg %. After removal of the second kidney these animals survived from 132 to 235 hours, averaging 190 hours. Before these animals died they showed marked electrocardiographic changes and toxic plasma potassium levels. Three of these animals received 20 mg DCA subcutaneously daily post-operatively, while 3 did not. There was no difference in the duration of life between these two sub-groups.

It was of interest to note that DCA lowered the plasma K in dogs with renal excretion, but did not affect the rate of accumulation of potassium in the plasma of binephrectomized dogs. This suggests that DCA operates via the kidney in lowering the plasma K in normal

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[9] Perlow, S., Killian, S. T., Katz, L. N., and Asher, R., *Am. J. Physiol.*, 1941, **134**, 755.

[10] Katz, L. N., Killian, S. T., Asher, R., and Perlow, S., *Am. J. Physiol.*, 1942, **137**, 79.

[‡] There was no correlation, with the doses used, between the amount of DCA given and life expectancy.

[11] Winkler, A. W., Hoff, H. E., and Smith, P. K., *J. Clin. Invest.*, 1941, **20**, 119.

[12] Ferrebee, J. W., Parker, D., Carnes, W. H., Gerity, M. K., Atcheley, D. W., and Loeb, R. F., *Am. J. Physiol.*, 1941, **135**, 230.

dogs, rather than acting on the extra-renal tissues. These results are in agreement with the studies of Durlacher and Darrow.[13]

Binephrectomy in two stages with fluid administration. The clinical condition of the animals in the foregoing experiments indicated that dehydration might be a contributory lethal factor. After removal of the second kidney, the effect of 50 cc daily of intravenous isotonic saline, 25% glucose in 50 cc isotonic saline, or 25% glucose in distilled water was tested on 2 dogs each. The average duration of life for the 3 series was 120, 83, and 154 hours respectively.

Blood Changes in Total Anuria. Aside from the changes in plasma potassium discussed above, certain other changes were observed. The plasma protein concentration, as indicated by the specific gravity of the plasma,[14] showed increases from the normal of 6 g % to as much as 10.4 g % on the sixth anuric day, thus giving confirmatory evidence of dehydration. The blood smear showed no significant changes.

The rate of accumulation of excretory products other than potassium was essentially the same in all groups of anuric dogs. The blood NPN (see [15] for method), averaging 35 mg % at the time of induction of anuria, increased 30 to 50 mg % per day.[16] Serum inorganic phosphorus (see [17] for method) was frequently found to increase from its normal of 4 mg to values as high as 18 mg % on the fourth anuric day.

Other constituents of the blood not measured by us, which have recently been reported to increase progressively after ablation of the kidneys, include total fatty acids, free and esterified cholesterol, phospholipids, free fat of serum,[18] phenol-like compounds,[19] and guanidine (Mason, quoted in [20]).

Cardiovascular changes. The heart rate did not change appreciably except terminally, when there was usually an increase in rate. Blood pressure changes were inconstant.

Limb lead electrocardiogram showed S-T depressions in leads I and II within 36 to 48 hours after induction of anuria. The T waves in all the leads flattened and became inverted or diphasic within 48 to 72 hours. After this the magnitude of the inverted T waves increased progressively and became notched. Among the other cardiac disturbances which developed progressively were auricular standstill or auricular fibrillation, intraventricular block and finally irregular ventricular tachycardia and ventricular fibrillation. These changes in the electrocardiogram are consistent with those seen in animals with progressive artificial increase in plasma potassium.[1] However, this does not necessarily exclude the participation of other mechanisms for their occurrence in anuria.

Pathological Changes. In 12 of the 59 animals studied, a striking lesion was noted in the ventral portion of the pulmonary artery in the region of the ductus arteriosus. The early stages of this lesion showed hemorrhagic areas in the adventitia. In more advanced cases there was involvement of the media with fibrinoid degeneration. The intima, while intact, frequently had a washboard appearance because of shrinkage of the subjacent media. Lesions similar to these have been described by Winternitz[21] following both binephrectomy and renal artery constriction.

Summary. 1. Technics for prolonging the life of the binephrectomized dog are given. 2. Some chemical, physiological, and anatomic changes seen in these anuric animals are described.

I am indebted to Dr. L. N. Katz, under whose guidance this study was carried out, for his con-

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[13] Durlacher, S. H., and Darrow, D. C., *Am. J. Physiol.*, 1942, **136**, 577.

[14] Barbour, H. G., and Hamilton, W. F., *J. Biol. Chem.*, 1926, **69**, 625.

[15] Peters, J. P., and Van Slyke, D. D., *Quantitative Chemical Chemistry*, Baltimore, 1932, Williams and Wilkins Co., Vol. II, p. 525.

[16] Bergman, H. C., and Drury, D. R., *J. Clin. Invest.*, 1939, **18**, 777.

[17] Bodansky, A., *J. Biol. Chem.*, 1932, **99**, 197.

[18] Winkler, A. W., Durlacher, S. H., Hoff, H. E., and Man, E. B., *J. Exp. Med.*, 1943, **77**, 473.

[19] Roen, P. R., *J. Urol.*, 1944, **51**, 110.

[20] Harrison, T. R., and Mason, M. F., *Medicine*, 1937, **16**, 1.

[21] Winternitz, M. C., and Waters, L. L., *Yale J. Biol. and Med.*, 1940, **12**, 451.

tinuous interest and advice. I also wish to express my gratitude to Dr. O. Saphir of the Department of Pathology for preparing and interpreting microscopic sections and to Dr. D. J. Cohn of the Department of Chemistry for some of the chemical determinations.

15035

Nitrogen and Sulfur Content of Liver Tissue in Relation to Carbon Tetrachloride Exposure.

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Present interest in the use of methionine in the therapy of acute chlorinated solvent poisoning[1, 2] stems to a large extent from the work of Miller and Whipple,[3] who demonstrated that this amino acid, given either shortly before or within 4 hours after the period of inhalation, would protect protein-depleted dogs against the otherwise fatal effects of a chloroform anaesthesia. Coincident with these results were the findings that protein-depleted dogs have livers which are deficient in both nitrogen and sulfur. Sulfur, however, is depleted more than is nitrogen, and therefore the N/S ratio rises. The ratio of nitrogen to sulfur in the liver tissue of their normal adult dogs was 11 or 12 to 1. The protein-depleted dogs which were so susceptible to liver injury showed a nitrogen to sulfur ratio of 14 or 15 to 1, which presumably means a lower content of sulfur-containing amino acids.

That an animal may be rendered inordinately susceptible to the vapors of chlorinated solvents or to hepatotoxic agents in general by manipulation of certain constituents of his diet is unquestioned. The principal point at issue in this investigation is whether inhalation of a chlorinated solvent produces any specific demand for sulfur-containing amino acids upon the animal already receiving an adequate protein intake. If such is the case, one might expect to find a significant depletion of liver sulfur reserves during the course of repeated exposures to the solvent, similar to that found over a period of maintenance on a protein-deficient diet. The effect of repeated inhalation of carbon tetrachloride vapor on the nitrogen and sulfur content of rat liver has been studied with the results presented below.

Experimental. The subjects of this investigation were 40 albino rats averaging about 200 g in weight. They were fed the basal diet described by White and Jackson.[4] The content of sulfur-containing amino acids in this diet is purposely limited; nevertheless, according to these authors, the quantity and nutritional quality of the protein are sufficient to support good growth for relatively long periods of time. After an initial 2-week period on this diet, one-half of the animals were subjected to 14 successive daily exposures, 6 hrs. per day, to 1000 p.p.m. of carbon tetrachloride vapor. The remaining half (controls) were unexposed. As the exposures caused some reduction in food intake, the controls were limited to the average food consumption of the exposed animals of the preceding day.

At the conclusion of the 2-week period of exposures all animals were sacrificed. The livers were removed, blotted free of excess blood, and dried for several days at 76°-78°C to constant weight. The dried livers were crushed to a fine powder, weighed into tared paper thimbles and extracted continuously with ethyl ether for 12 hours in a Bailey-

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[1] Beattie, J., Herbert, P. H., Wechtel, C., and Steele, C. W., *Brit. Med. J.*, 1944, No. 4336, 209.

[2] Eddy, J. H., Jr., *Ind. Med.*, 1945, **14**, 283.

[3] Miller, L. L., and Whipple, G. H., *J. Exp. Med.*, 1942, **76**, 421.

[4] White, A., and Jackson, R. W., *J. Biol. Chem.*, 1935, **111**, 507.

TABLE I.
Nitrogen and Sulfur Content of Dry, Fat-Free Liver Tissue from 20 Control Rats and 20 Rats Receiving 14 Successive Daily Exposures to 1000 p.p.m. CCl_4.

Group	% Sulfur		% Nitrogen		N/S	
	Avg	Std. error	Avg	Std. error	Avg	Std. error
Controls	0.903	±0.188	10.672	±0.939	12.281	±1.758
Exposed	1.105	±0.163	12.348	±1.661	11.408	±1.390
Difference	0.202	±0.058	1.676	±0.424	0.873	±0.53

Walker apparatus. The decrease in weight after extraction was calculated as percentage of fat. Total nitrogen content of the dry, fat-free residues was estimated by macro-Kjeldahl, and total sulfur by a wet-ashing method.[5]

Results. As was to be expected, the livers of the exposed animals exhibited gross fatty infiltration. The sulfur content of the fat-free liver tissue from these animals, far from showing a depletion, was actually greater than that of the unexposed animals (Table I). Coincidently, total nitrogen of the former was also elevated, so that the nitrogen to sulfur ratios were not significantly different.

Comment. The failure of repeated inhalation of carbon tetrachloride to effect some diminution in liver sulfur in animals receiving an adequate protein intake argues against a specific role of the sulfur-containing amino acids in protection of the liver from acute chlorinated solvent poisoning. The greater susceptibility of animals receiving protein—and/or methionine—deficient dietaries to these solvents continues to be explained adequately by the observed facts that (a) a deficiency of lipotropic factors creates a specific type of hepatic injury *per se*,[6] and that (b) these dietaries tend to produce livers containing maximal concentrations of fat.[7] These considerations, of course, do not obtain in the normal animal, so that the employment of methionine therapy in his case remains to be justified. This in no way discounts the value of a high-protein diet in the treatment of liver injury, since protein itself is of obvious importance in the repair of structural damage.

Summary. Values for total nitrogen and total sulfur determined on the dry, fat-free liver tissue of rats following repeated exposure to carbon tetrachloride vapor were slightly higher than those of rats maintained on the same diet, but not exposed. The nitrogen to sulfur ratios of the two groups, however, were not statistically different. The significance of these findings is discussed in relation to the general problem of the protection of the liver from toxic agents by sulfur-containing amino acids.

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[5] Evans, R. J., and St. John, J. L., *Ind. Eng. Chem., Anal. Ed.*, 1944, **16**, 630.

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15036

Relation Between Thiamine and Co-Carboxylase Concentration and Work Output of Perfused Frog Muscles.*

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The addition of 0.01 mM of thiamine hydrochloride per liter of perfusion fluid has been shown to increase significantly the work output of perfused frog muscles.[1] Observations on the relationship between thiamine concentrations in the perfusing fluid and the increase in work output are presented in the following report. In addition, the effectiveness of pyrophosphate thiamine or co-carboxylase in augmenting work output in perfused frog muscles has been assessed.

Experimental. The experimental methods used have been described in detail in a previous report.[1] Summarized briefly, fatigue curves were recorded for each gastrocnemius muscle of the frog under comparable conditions of perfusion, stimulation, loading, and recording. In the present experiments, stimulation was effected by single shocks from a Bird Electronic Inductorium operating through a voltage regulating transformer. Thiamine hydrochloride or co-carboxylase was added to the buffered glucose-Ringer's solution used for perfusion of the frog while the fatigue curve was recorded on the second gastrocnemius. Ten animals were tested with each concentration of thiamine or co-carboxylase. Thiamine concentration of the final perfusion fluid was varied from 0.0001 mM per liter to 1.5 mM per liter by adding appropriate amounts of a stock standard solution of thiamine containing 100 mM per liter. Co-carboxylase concentrations of 0.00001 to 0.1 mM per liter were prepared from a stock standard solution containing 50 mM per liter.[†] Individual work curves were plotted for each experiment and the total work done was estimated from the areas under the curves as determined with a planimeter.

Results. Effect of increasing concentration of thiamine on work output. The effect of adding 0.01 mM thiamine hydrochloride to the perfusing fluid is illustrated in Fig. 1. Fatigue curve A was first recorded from the left gastrocnemius muscle of a frog perfused with a buffered glucose Ringer's solution. The fatigue curve B was obtained on the right leg of the same animal after 15 minutes perfusion with the same buffered glucose Ringer's solution in which the thiamine concentration was .01 mM per liter. A marked improvement in work output of the muscle receiving thiamine is apparent. Total work output was calculated for each experiment as described previously,[1] and averages computed. The average difference in work output of the control and experimental legs obtained in 10 animals was calculated with its standard error.[‡] Table I shows the average total work done by control and experimental legs with increasing concentration of thiamine. This table shows

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[*] With the technical assistance of Frances Berg. A preliminary report was presented to Section N of the American Association for the Advancement of Science at the meeting held in Cleveland, September 10-11, 1944.

[1] Shock, N. W., and Sebrell, W. H., *Am. J. Physiol.*, 1944, **142**, 265.

[†] Thiamine hydrochloride and thiamine pyrophosphate, Merck, were used in preparing the stock solution by weight.

[‡] The mean difference (experimental-control) was calculated for each group of 10 animals. The standard error of the mean difference was calculated from the distribution of differences as

$$\sigma_{\text{Mn. Diff.}} = \frac{\sqrt{\frac{\Sigma(\text{Exp.-Cont.})^2}{N} - (\text{Mn. Diff.})^2}}{\sqrt{N-1}}$$

and the critical ratio as

$$\text{C. R.} = \frac{\text{Mn. Diff.}}{\sigma_{\text{Mn. Diff.}}}$$

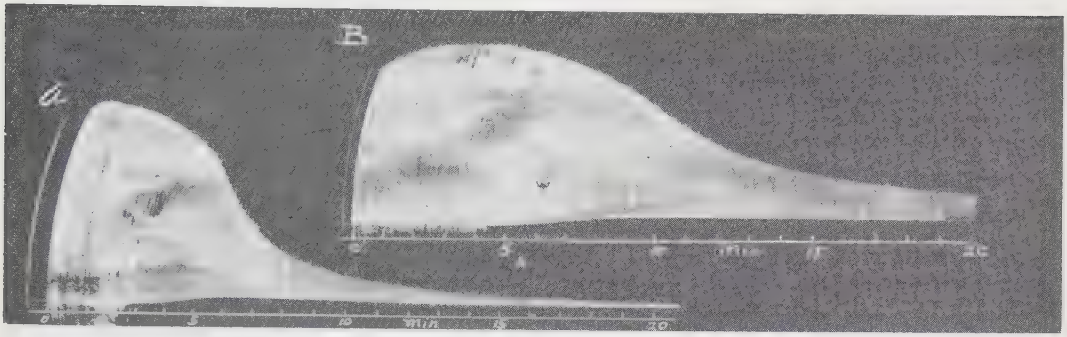

FIG. 1.

Improvement in work output of gastrocnemius muscle of perfused frog following the addition of 0.01 mM thiamine hydrochloride to perfusion fluid. Fatigue curve A of right gastrocnemius muscle during perfusion with buffered glucose-Ringer's solution. Fatigue curve B recorded from left leg of same frog after 15-minute perfusion with buffered glucose Ringer's solution containing 0.01 mM thiamine hydrochloride per liter. Time in minutes.

TABLE I.

Effect of Thiamine Concentration on Total Work Output of Perfused Frog Muscles.

Conc. of thiamine HCl mM/L	Avg work done (n = 10 animals)			C.R.†
	Cont. leg g cm	Exp. leg g cm	Mean difference* (Exp.-Cont.) g cm	
0.00 (a)‡	1449	1596	147 ± 93.0	1.6
0.00 (b)	2271	2421	150 ± 112.1	1.3
0.00 (c)	2302	2454	152 ± 73.0	2.0
0.0001	1793	1899	106 ± 69.7	1.5
0.0002	1884	2025	141 ± 80.5	1.8
0.0003	2177	2351	174 ± 122.8	1.4
0.0005	2281	2522	241 ± 87.5	2.8
0.0007	1824	2138	314 ± 103.6	3.0
0.00075	2315	2759	444 ± 36.0	12.3
0.001 (d)	2010	2428	418 ± 83.0	5.0
0.001 (e)	1553	1889	336 ± 95.1	3.5
0.005	1281	1538	257 ± 73.1	3.5
0.01	2042	2443	401 ± 97.3	4.1
0.1	2490	2823	333 ± 136.7	2.4
0.25	1958	2259	274 ± 86.6	3.2
0.5	2511	2868	357 ± 64.6	5.5
0.75	2533	2819	286 ± 129.4	2.2
1.0	2241	2460	219 ± 32.7	6.7
1.5	2196	2388	192 ± 57.5	3.3

* ±1 Standard error of the mean.

† Critical ratios greater than 3.0 are considered statistically significant. With 10 pairs of observations this criterion is equivalent to a P of about 0.01, which means that in only 1 out of 100 trials would one expect a difference as large or larger than the observed one to occur by chance (based on t table for small numbers).

‡ Groups a, b, c, d, and e as indicated in Discussion.

that the increase in work output observed at thiamine concentrations less than 0.0005 mM per liter were not statistically significant. Thiamine concentrations greater than 0.0005 mM per liter resulted in a significant improvement in work output of the treated muscle.

In Fig. 2 the average percentage increase in work output of the treated muscle over its untreated control is plotted against the logarithm of the concentration of thiamine in the perfusing fluid. This figure shows that the work output increases when the concentration of thiamine is increased from 0 to 0.001 mM per liter. With thiamine concentrations of

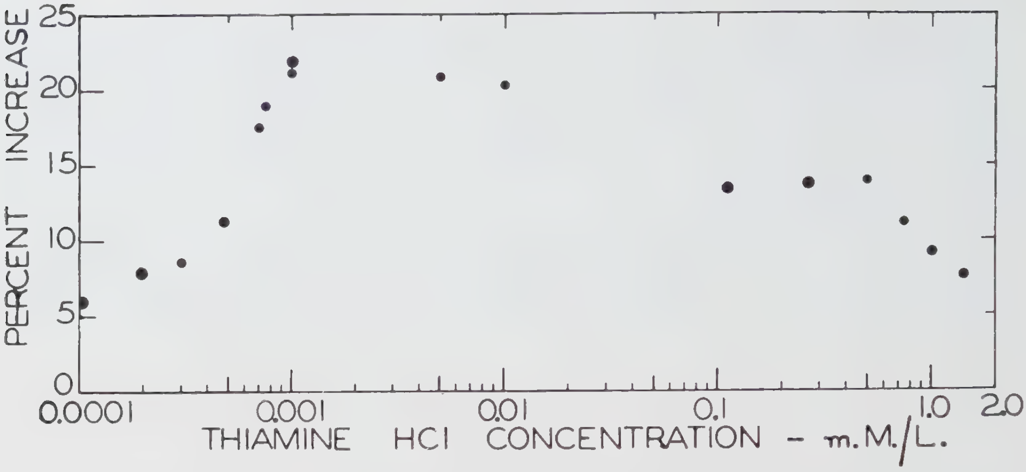

Fig. 2.

Effect of increasing concentrations of thiamine hydrochloride in the perfusion fluid on improvement in work output of frog muscles. Abscissa—thiamine hydrochloride concentration in perfusion fluid, mM per liter. Ordinate—% increase in total work done by muscle perfused with Ringer's solution plus thiamine overwork done by muscle perfused with Ringer's solution alone. Each point is the average of 10 experimental animals. Differences greater than 10% are statistically significant.

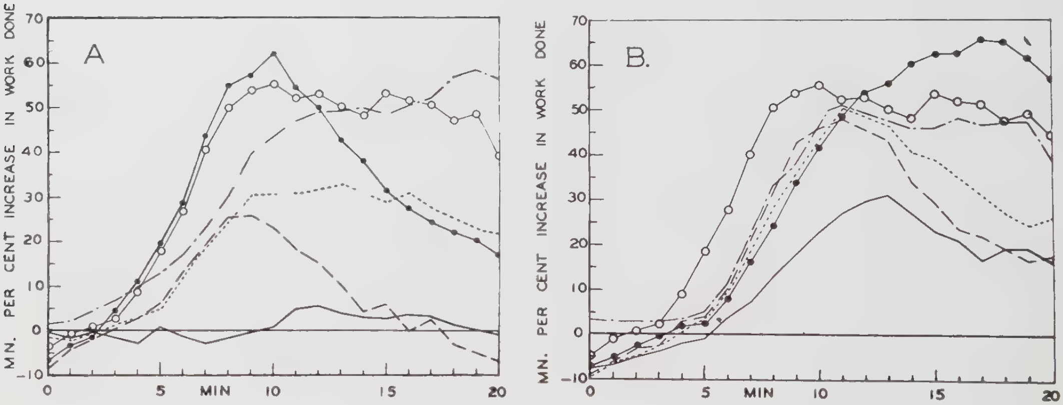

Fig. 3.

Effect of increasing concentrations of thiamine hydrochloride in perfusion fluid on distribution of work outside of frog muscles.

A. ———— Control (no vitamin added to perfusion fluid of second muscle); —— —— —— 0.0002 mM/L thiamine HCl; - - - - - - - - - - - - 0.0005 mM/L thiamine HCl; ——— — ——— 0.00075 mM/L thiamine HCl; ●—●—●—● 0.001 mM/L thiamine HCl; ○—○—○—○ 0.01 mM/L thiamine HCl.

B. Thiamine HCl concentrations of: ○—○—○—○ 0.01 mM/L; ●—●—●—● 0.1 mM/L; ——— - ——— 0.5 mM/L; - - - - - - - - - - - - 0.75 mM/L; —— —— —— 1.0 mM/L; ———— 1.5 mM/L.

Each point is the average of 10 experiments. Differences greater than 15% are statistically significant.

0.001 to 0.1 mM per liter, the maximum improvement in work output is obtained. At higher thiamine concentrations (0.1 to 1.5 mM per liter) the improvement in work output diminishes. Statistical tests showed that in this series of experiments differences in total work output greater than 10% were statistically significant. (See Table I.)

The average percent differences between the height of contraction of the control and vitamin-treated muscles at each minute of exercise are plotted in Fig. 3-A and B. Fig. 3-A shows that as the concentration of thiamine is increased from 0.0002 to 0.01 mM per liter

TABLE II.
Effect of Thiamine Pyrophosphate (Co-carboxylase) on Work Output of Perfused Frog Muscle.

		Avg work done (N = 10 animals)			
Substance[*]	Conc. mM/L	Cont. leg g cm	Exp. leg g cm	Mean difference[†] (Exp.-Cont.) g cm	C.R.[‡]
TP	0.00001	1834	1984	150 ± 73.5	2.0
TP	0.0001	1905	2213	308 ± 60.5	5.1
TP	0.001	1928	2175	247 ± 47.8	5.2
TP	0.01	2072	2348	276 ± 74.3	3.7
TP	0.1	1904	2196	292 ± 75.7	3.9
TP[§]	0.01	2134	2348	214 ± 49.9	4.3
THCl[§]	0.01	2107	2286	179 ± 34.2	5.2
THCl + TP[‖]	0.01	1977	2188	211 ± 45.0	4.7
THCl + TP[‖]	0.01	1855	2074	219 ± 56.0	3.9
TP + THCl[¶]	0.01	2212	2321	109 ± 87.5	1.2
TP + THCl[¶]	0.01	1993	2069	76 ± 52.9	1.4

[*] TP = Thiamine pyrophosphate. THCl = Thiamine hydrochloride.

[†] ±1 Standard error of the mean.

[‡] Critical ratios greater than 3.0 are statistically significant.

[§] Second leg (experimental) perfused with vitamin for only 5 minutes before running fatigue curve instead of 15 minutes as in all other experiments.

[‖] Control leg perfused with buffered glucose-Ringer's solution containing 0.01 mM/L thiamine hydrochloride. Experimental leg perfused with the same mixture plus 0.01 mM/L thiamine pyrophosphate.

[¶] Control leg perfused with buffered glucose-Ringer's solution containing 0.01 mM/L thiamine pyrophosphate. Experimental leg perfused with the same mixture plus 0.01 mM/L thiamine hydrochloride.

the work output is increased significantly during the later stages of exercise. Fig. 3-B shows that when the thiamine concentration in the perfusing fluid is increased from 0.1 mM per liter to 1.5 mM per liter the improvement in work output diminishes. In Fig. 3-A and B, differences greater than 15% are statistically significant.

Effect of thiamine pyrophosphate (co-carboxylase) on work output. Table II shows the work output when thiamine pyrophosphate was added to the perfusion fluid in concentrations of 0.1 to 0.00001 mM per liter. A significant increase in total work was observed when concentrations greater than 0.00001 mM thiamine pyrophosphate per liter were added to the perfusion fluid. At concentrations greater than 0.001 mM per liter, the increment in work output did not differ significantly from that observed with similar concentrations of thiamine (see Table I). However, the thiamine pyrophosphate was effective at lower concentrations than was thiamine hydrochloride.

Experiments were also performed to see whether the physiological response to thiamine pyrophosphate appeared more rapidly than the response to thiamine alone. For this purpose, the time of perfusion with the vitamin was reduced from 15 to 5 minutes in a series of 10 experiments. The results appear in lines 6 and 7 of Table II, which show that a significant increase in work output can be obtained within 5 minutes with both thiamine pyrophosphate and thiamine hydrochloride. Although the work output was greater with thiamine pyrophosphate than with thiamine hydrochloride, the difference is not statistically significant.

The combined effect of thiamine pyrophosphate and thiamine hydrochloride was tested in the following manner. In one set of 10 experiments, thiamine hydrochloride (0.01 mM/L) was added to the buffered glucose Ringer's solution used to perfuse the first or control leg. Thiamine pyrophosphate was added to this solution for perfusion of the second or experimental leg. In another set of 10 experiments, the order of adding the hydrochloride and the pyrophosphate was reversed; *i.e.*, thiamine pyrophosphate was added to the perfusion fluid used for the first or control leg, and thiamine hydrochloride was then added to the perfusion fluid used for the second or experimental leg. The results of these experiments appear in the last four lines of Table II. These experiments, repeated in duplicate, showed that a significant increase in work output was obtained from the addition of thiamine pyrophosphate (0.01 mM/L) to a muscle already treated with an optimum concentration of thiamine hydrochloride. On the contrary, no significant improvement in work output followed the addition of thiamine hydrochloride to a muscle already exposed to an adequate concentration of thiamine pyrophosphate.

Discussion. The results in Table I show that the total work output varies considerably between different groups of frogs. For instance, the average work output of Group a (obtained in March) is considerably less than that of Groups b or c (obtained June and September, respectively). Nevertheless, the differences between the work output of the two legs is of the same order of magnitude. Similarly, the increment in work output obtained after the administration of thiamine is about the same, as shown by duplicate experiments with 0.001 mM thiamine per liter (d and e of Table I; d—runs made in June, e—runs made in November). The large standard errors of the mean differences between work output of the two legs (column 5, Table I) shows that some animals are affected to a much greater degree than others by the administration of the thiamine. The source of this variability in response is not clear from the available data.

The data of Fig. 1 show that the most effective concentration of thiamine for improving work output is 0.001 mM per liter, as compared with 0.01 mM per liter for calcium pantothenate.[2] The maximum improvement in work output is practically the same for each vitamin. Since the normal mammalian blood levels reported in the literature are 0.0003 mM per liter for thiamine[3,4,5,6] as compared with 0.001 mM per liter for pantothenic acid, it is not surprising to find that thiamine improves work output in lower concentrations than does pantothenic acid.[2] The most effective concentration of thiamine was thus 10 to 100 times the normal blood levels.

Evidence that the improvement in work output observed after the administration of thiamine is associated with metabolic processes in the muscle itself is afforded by the observation of improved work output when thiamine pyrophosphate is added to a muscle already receiving an adequate amount of thiamine hydrochloride. The available evidence is inadequate to decide whether this difference is due to differences in permeability of the phosphate and hydrochloride, or to the rate at which the hydrochloride is phosphorylated in the muscle tissue, since it is presumed that thiamine is effective in muscle energy metabolism chiefly as the pyrophosphate—or co-carboxylase.[7]

The present experiments show that maximum improvement in work output occurs with concentrations of thiamine between 0.001 and 0.1 mM per liter. With concentrations greater than 0.1 mM, there is a progressive diminution in effectiveness. A similar phenomenon was observed with calcium pantothenate.[2] Since the pH was maintained at 7.4 even with 1.5 mM per liter thiamine, changes in acidity cannot account for the diminution in physiological effect. Although the data are incomplete, Briem[8] has reported a reduction in contractility of frog muscles at high concentrations of thiamine. Previous investigations have also indicated an increase in the strength of the heart beat at low thiamine concentrations, where high concentrations caused cardiac arrest in the frog.[8,9] Schneider[10] has observed a lethal effect of high vitamin B complex on *Tribolium confusum* Duval in contrast to the growth-promoting effect of low concentrations of the same complex. While the mechanism whereby an excess of thiamine or pantothenate may fail to improve work output is problematical, the diminished effect of high concentrations is apparently real.

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Summary. The work output of the gastrocnemius muscle of the perfused frog has been determined at different levels of thiamine concentration in the perfusion fluid. The effects on the work output of adding thiamine pyrophosphate (co-carboxylase) and thiamine hydrochloride to the perfusion fluid has been compared. 1. The addition of thiamine hydrochloride to the perfusion fluid improved the work output of frog muscles. The degree of improvement increased with increasing concentrations of hydrochloride from 0.0001 to 0.001 mM per liter. 2. No further improvement in work output resulted when thiamine hydrochloride concentration was increased to 0.1 mM per liter. 3. Increasing the thiamine concentration of the perfusion fluid from 0.1 to 1.5 mM per liter progressively reduced the improvement in work output. 4. Thiamine pyrophosphate was also effective in increasing the work output in perfused frog muscles. 5. Thiamine pyrophosphate increased the work output at lower concentrations than did thiamine hydrochloride. 6. At concentrations of 0.001 mM per liter or above, the extent of the improvement in work output was no greater with thiamine pyrophosphate than with thiamine hydrochloride.

15037

X. Carcinoma of Mammary Gland Following Injection of Methylcholanthrene into Mice of NHO Strain.

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Adenocarcinomas of the mammary gland in mice have been known since 1854.[1] The usual degree of morphologic variation exhibited by mouse mammary gland tumors has been interpreted by Apolant,[2] and others,[3] as representing a series of sequential phases of a single pathological process. A continuous gradient may be observed from the benign adenomas to the adenocarcinomas and the undifferentiated medullary carcinomas. In general these tumors are only histologically malignant, with remote metastases and extensive invasion of adjacent tissues occurring only infrequently.

Numerous agents have been incriminated by the many investigators of the problem as having some influence on the origin of these tumors in mice. However, 5 influences have received rather widespread recognition. These influences may be briefly classified as (1) genetic; (2) hormonal; (3) the "milk" influence; (4) dietary factors; and (5) somatic mutation.

Recently mammary gland tumors which had been encountered only in genetically susceptible mice, either spontaneously or following hormone injection, have been duplicated following the subcutaneous injection of methylcholanthrene in sesame oil into female mice of certain strains which lack the milk influence, are spontaneously resistant to mammary gland tumors, and do not develop such tumors after estrogen treatment.[4,5,6] This observa-

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tion has been verified in several laboratories with various strains of resistant mice.[7,8,9,10,11]

In this investigation, mice of the NH and NHO strains have been used since they are known to be genetically resistant to mammary gland tumors. There has never been obtained a single case of mammary tumor in the control NH mice (under both breeding and non-breeding regimes), nor in the untreated descendants of methylcholanthrene-treated animals. Mice of the NH and NHO strains are, therefore, up to the present time, highly resistant to spontaneous breast tumors. This conclusion is based upon the following unpublished data: 476 virgin female mice of the original control NH stock autopsied between 150 and 820 days; 190 female mice used as breeders of the same control NH stock autopsied between 180 and 730 days; and 431 untreated female descendants of methylcholanthrene-treated mice autopsied between 150 and 730 days (375 non-breeders and 56 mice that had been used for breeders). In addition to these observations, it has been determined that, in an outcross of the original control NH stock mice to mice of the C_3H strain, spontaneous tumors of the mammary gland occur only in the F_1 female mice provided the mother came from the cancer-susceptible C_3H strain (maternal inheritance). It has been found out too (unpublished data) that when C_3H female mice are fostered onto NH mothers, they do not develop spontaneous tumors of the mammary gland.

Originally mammary gland tumors occurred sporadically following the injection of methylcholanthrene. At present, however, mammary tumors can be induced in at least 60% of the female mice of certain strains treated with the carcinogen. This was the incidence obtained in two sublines of the NHO strain[12] which had been genetically selected toward suppression of local methylcholanthrene-induced tumors such as fibrosarcoma, epidermoid carcinoma and rhabdomyosarcoma.[13] In the most recent observations on mice of two sublines of this selected strain, which were in the F_{14} to F_{18} generations following an outcross, 75 female mice out of a total of 125 injected subcutaneously with methylcholanthrene developed mammary gland tumors, either locally at the site of injection or in any other of the mammary gland regions.

These tumors induced by methylcholanthrene duplicate in every histologic detail the mammary tumors which occur in mice either spontaneously or after the injection of an estrogenic hormone. In addition to the usual histology, however, many of the tumors possess certain new characteristics such as metastasis to bone and invasion of adjacent tissues. Anaplasia and squamous metaplasia are observed with greater frequency than in tumors of the mouse mammary gland obtained by earlier means.

Upon examination of a typical example among the more undifferentiated tumors, it may be noted that the morphology varies considerably in different portions of a single tumor. In some areas the stroma is scanty, in others abundant. The tumor cells are hyperchromatic and may either be packed densely in solid sheets or occur singly or in small clumps in the abundant stroma. All traces of an acinar origin have been lost. Frequently, elongated cords of nearly spindle-shaped tumor cells, sometimes a single cell layer thick, may be observed. The tumor invades early the surrounding tissue and in some areas there seems to be no capsule. The tumor cells that invade the surrounding musculature and the neighboring bony structures are very anaplastic. The metastatic nodules in bone erode the cortex and fill the medullary cavity, replacing the bone marrow. The mammary gland in the neighborhood of one of these ana-

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TABLE I.

Includes the Autopsy Records on Female Mice of the NH and NHO Strains in Reference to Mammary Gland Tumors. Mel. means 1 mg of methylcholanthrene dissolved in 0.1 ccm sesame oil and injected subcutaneously at 60 days.

Strain	Class	Generations inbreeding	Type female	No. mice	Mammary tumors	Autopsy age range
NH	Control	F_{10}-F_{20}	Breeder	198	0	180-730
		F_{10}-F_{20}	Virgin	476	0	150-820
NHO	Untreated descendants	F_{10}-F_{18}	Breeder	56	0	150-730
		F_{10}-F_{18}	Virgin	375	0	150-730
NHO	Injected	F_{14}-F_{18}	Breeders	125	75	84-525
Total controls				1105	0	
Exp. (Mel. inj.)				125	75	

plastic tumors appears to be quite hyperplastic and undergoing squamous metaplasia.

Several conclusions may be drawn from the results of this investigation to date:

1. The series of breast tumors occurring under spontaneous conditions in genetically susceptible female mice and following the injection of estrogenic hormones in male mice can be duplicated in apparently resistant female mice of certain strains following the subcutaneous injection of methylcholanthrene.

2. The injection of methylcholanthrene can therefore replace spontaneous genetic susceptibility. These mice may be genetically susceptible to this particular agent (methylcholanthrene) even though resistant to others but the manner of inheritance has not, as yet, been ascertained. The suggestion should be entertained that the present experiment may represent evidence of agent-specificity of genetic control.

3. The tumors arising in female mice genetically resistant to spontaneous mammary tumors following the injection of methylcholanthrene possess 4 characteristics not present or less pronounced in the tumors of genetically susceptible female mice: (a) they show an increased tendency to undergo squamous metaplasia; (b) many of them (approximately 18%) show rather pronounced anaplastic changes; (c) these anaplastic tumors invade surrounding normal tissues rather extensively and (d) do metastasize to bone.

4. These malignant breast tumors in mice appear closely to simulate some of the characteristics of human mammary carcinomas.

Much further work is necessary on this phenomenon of the production of anaplastic carcinoma, including an investigation of the roles of the various agents, such as the milk influence and estrogens, which are known to influence the other types of tumors of the mammary gland in mice. It is hoped to demonstrate whether the action of methylcholanthrene actually involves a reversal of genetic resistance or merely permits the manifestation of a new type of agent-specific susceptibility which will not spontaneously yield mammary tumors.

Summary. 75 female mice of a total of 125 injected subcutaneously with 1 mg of methylcholanthrene at 60 days of age, developed carcinomas of the mammary gland. The mice belonged to the F_{14}-F_{18} generations of inbreeding. This high incidence of mammary gland tumors was made possible by the suppression of local tumors through the process of genetic selection toward resistance to such local tumors. These induced mammary gland tumors duplicate the series of tumors produced spontaneously in genetic susceptible strains of mice such as the A and the C_3H. In addition, however, the tumors arising in genetically resistant female mice of this experiment following the injection of methylcholanthrene possess 4 characteristics not present or less pronounced in the tumors of genetically susceptible female mice: (a) they show an increased tendency to undergo squamous metaplasia; (b) many of them (approximately 18%) undergo rather pronounced anaplastic changes, (c) these anaplastic tumors

invade surrounding normal tissues rather extensively and (d) do metastasize to bone. These new malignant breast tumors in mice appear closely to simulate some of the characteristics of human mammary carcinomas.

15038

Effect of p-Aminobenzoic Acid on Growth of Rickettsiae and Elementary Bodies, with Observations on Mode of Action

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In a previous report,[1] publication of which was delayed for reasons of military security, it was shown that the growth of typhus rickettsiae in the yolk sac of the developing chick was markedly inhibited by p-aminobenzoic acid.* We demonstrated further that the best inhibition of rickettsial growth was obtained only when maximal dosages of paba were used, which in the case of the chick embryo was 2-4 mg per egg. The lowest amount of paba which would give a certain inhibition was 0.2-0.4 mg per egg. These experiments were an outgrowth of a preliminary report by Snyder, Maier, and Anderson[2] in which data (on mice infected with murine typhus) were presented which suggested that paba might be of therapeutic value. On the basis of the fact that maximal dosages of paba are necessary for the inhibition of rickettsiae, large amounts of paba were then administered to humans infected with typhus, and good therapeutic results were obtained (Yeomans *et al.*)[3] Greiff, Pinkerton, and Moragues[4] have studied the effect of enzyme inhibitors and activators (penicillin, para-aminobenzoic acid, sodium fluoride, and vitamins) on the multiplication of typhus rickettsiae, and have also found that paba inhibits rickettsial growth.

In our first report it was mentioned that a similar inhibition of rickettsial growth was obtained when eggs that had been infected with Rocky Mountain spotted fever were treated with paba. A more detailed analysis of the action of this drug on different members of the spotted fever group showed not only that the inhibition was more striking in many cases than was obtained with typhus, but that differences in magnitude of the response of different kinds of rickettsiae to paba could be correlated with their antigenic relationships. It is the purpose of the present report to present these quantitative aspects of the growth of different kinds of rickettsiae (and some elementary bodies) in the presence of paba. Data are also included which have a bearing on the possible mode of action of paba.

Methods. The methods used in measuring the effect of paba on the growth of rickettsiae were similar to those previously described.[1] Hen's eggs which had been incubated 8 days and contained normal-appearing, vigorous embryos were selected and divided into two lots. Each egg of the first, or control, lot received an injection of 0.5 cc of sterile distilled water in the yolk sac, and each egg of the test series received a similar injection of 0.5 cc of a sterile solution of paba in distilled water. Immediately after the injection with these solutions, each egg was infected by injecting

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TABLE I.

Influence of Paba on Time of Death of Chick Embryos Infected with Rickettsiæ of the Spotted Fever Group.

		Total No. of eggs		No. of daily deaths										
			Day:	3	4	5	6	7	8	9	10	11	Survivors	% dead
Rocky Mt. Spotted Fever	Controls	58		8	48	2	—	—	—	—	—	—	0	100
	Paba—2 mg	56		1	1	3	3	3	2	4	6	7	26	53.6
Brazilian Spotted Fever	Controls	72		2	47	22	1	—	—	—	—	—	0	100
	Paba—2 mg	59		0	0	1	0	3	4	13	13	15	10	83.1
Colombian Spotted Fever	Controls	75		3	56	16	—	—	—	—	—	—	0	100
	Paba—2 mg	63		0	0	1	1	4	19	13	12	8	5	92.1
South African Tick Fever	Controls	51		2	6	31	10	2	—	—	—	—	0	100
	Paba—2 mg	51		0	1	2	2	4	8	8	8	10	8	84.3
Fièvre Boutonneuse	Controls	63		7	24	27	5	—	—	—	—	—	0	100
	Paba—2 mg	59		3	2	9	9	8	13	8	4	2	1	98.3

0.5 cc of a suspension of the rickettsiae to be tested. The infectious material was obtained from infected yolk sacs by grinding one part of yolk sac with alundum and diluting with 25 parts of sterile skim milk. The eggs were then sealed with wax and incubated at 37.5°C. Daily recordings were made of the deaths of embryos, and smears from each yolk sac were stained and recordings made of the average number of rickettsiae seen in each oil-immersion field. In general, we have found that the time of death is a far more reliable measuring index than the number of rickettsiae in smears, because rickettsiae vary in number according to the length of time that the egg has been dead and even according to the portion of the sac which is sampled. Therefore, the results which are presented here are based entirely on time of the embryo's death as a measure of rickettsial growth.

A uniform dosage of 2 mg of paba per egg was used in all of these experiments except when otherwise stated.

Results. Rocky Mountain Spotted Fever: Under the conditions of culture just described, the rickettsiae of Rocky Mountain spotted fever are ordinarily very toxic for chick embryos, with most of the deaths occurring between the third and fourth days after infection. Smears from the yolk sac usually contain a small number of rickettsiae (1-50 rickettsiae per oil-immersion field). The rickettsiae of typhus, on the other hand, do not ordinarily kill the embryos until 5-6 days after infection, and smears from the yolk sac are often filled with countless numbers of rickettsiae. This difference in the yield of rickettsiae may be partly explained by the lesser toxicity and consequent longer growth period of the typhus rickettsiae.

When eggs are simultaneously infected with Rocky Mountain spotted fever and injected with a 2 mg dosage of paba, then the early and complete mortality, noted above, does not occur. Table I and Fig. 1 summarize the results obtained with 58 control and 56 paba-treated eggs. All of the control embryos died early of the infection, with an average day of death of 3.9. In contrast to the sharp endpoint with the controls, embryos which had received paba showed only scattered deaths, with over 46% surviving to the time of hatching (19th day of incubation). Smears from the yolk sacs of dead control embryos contained the usual moderate number of rickettsiae (1-50 per field), but, in most cases, no rickettsiae could be found in the smears made from paba-treated eggs. The few paba-treated eggs in which rickettsiæ could be demonstrated rarely contained more than a single rickettsia per field.

The minimum amount of paba which would cause a measurable prolongation of survival time was determined in a second series of experiments. Dilutions were made of the stock paba solution (4 mg/cc) so that the 0.5 cc dosage would contain different amounts of the drug. Control embryos received 0.5 cc amounts of distilled water. By a process of bracketing, within a concentration range of

Figure 1. Effect of Paba on Time of Death (Rocky Mountain Spotted Fever)

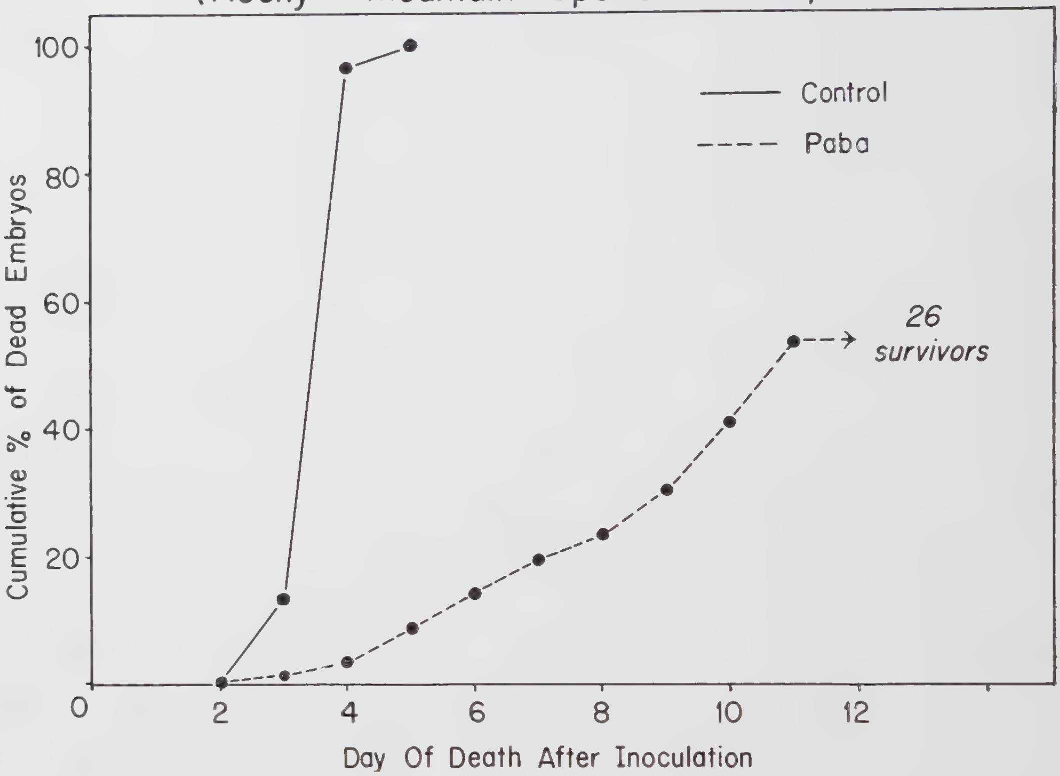
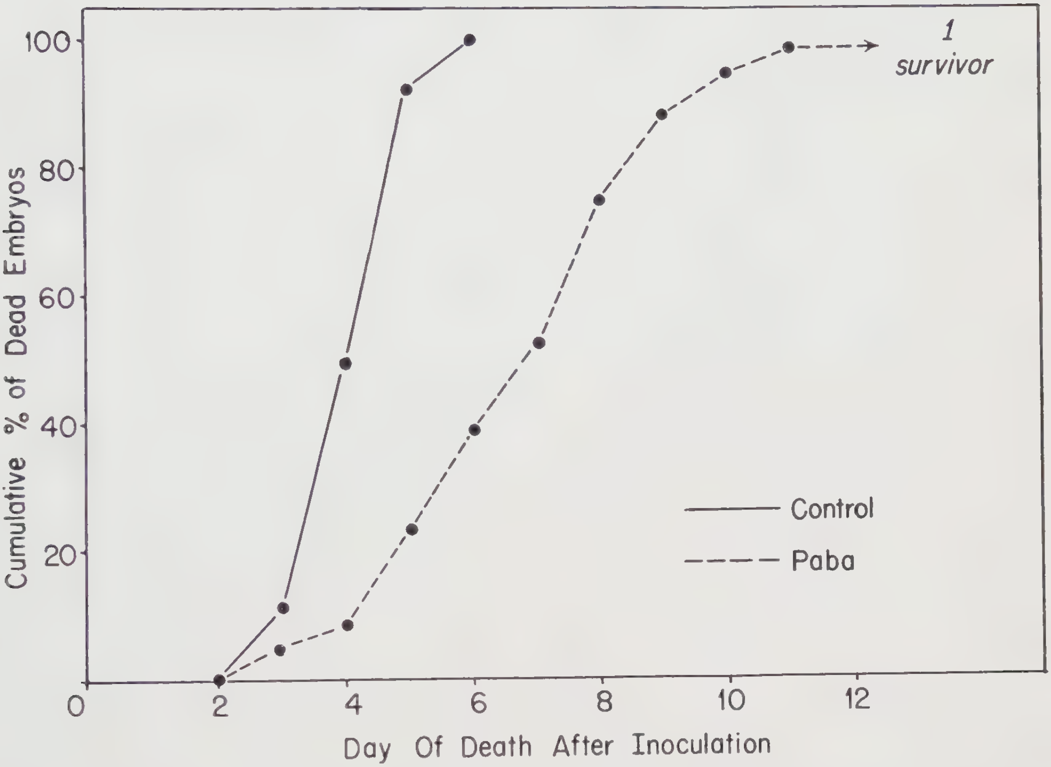


TABLE II.
Effect of Different Concentrations of Paba on Survival of Chick Embryos Infected with Rocky Mountain Spotted Fever.

	Concentration of Paba	Total No. of eggs	No. of daily deaths Day: 3	4	5	6	7	8	9	10	Avg day of death
Exp. 1	0 mg	8	1	6	1						4.0
	0.0625	8	2	4	2						4.0
	0.25	10	0	6	4						4.4
	1.0	9	1	0	0	0	0	0	7	1	8.4
Exp. 2	0 mg	9	1	6	2						4.1
	0.1	7	1	4	2						4.1
	0.25	12	0	4	8						4.7
	0.5	6	0	0	2	1	1	2			6.5
Exp. 3	0 mg	10	0	4	6						4.6
	0.1	13	1	5	7						4.5
	0.25	8	0	2	6						4.8
	0.5	9	0	0	4	3	2				5.8

0.0625-1.0 mg per egg, the minimal effectual concentration was found to be ±0.25 mg. The results of 3 separate experiments are presented in Table II. It will be noted that the average day of death was not significantly altered by a dosage of 0.1 mg or less, but that there was a consistent and appreciable increase in the average day of death with concentra-

Figure 2. Effect of Paba on Time of Death (Fièvre Boutonneuse)



tions of 0.25 mg or more. It is of interest that the number of rickettsiae seen in smears from treated eggs was inversely correlated with the amount of drug which was given. The minimal effectual dosage of paba, as determined for spotted fever (±0.25 mg), is of the same order of magnitude as that obtained with typhus rickettsiæ (0.2-0.4 mg.)[1]

Other Rickettsiae of the Spotted Fever Group: In view of the striking inhibitory action of paba on the growth of Rocky Mountain spotted fever rickettsiae, a survey was made of other members of the spotted fever group to see if differences could be detected in the magnitude of the inhibition. An identical technique was used for testing Brazilian spotted fever, Colombian spotted fever, South African tick fever, and fièvre boutonneuse, with results as shown in Table I. It will be noted that with each of these kinds of rickettsiae there was a prolongation of the survival time in the presence of paba, with some embryos surviving to hatching. Practically identical survival times were obtained with South African tick fever and Brazilian spotted fever, with 16% and 17% survivors, respectively. There were fewer survivors with Colombian spotted fever (8%) and fièvre boutonneuse (1.7%). With regard to the number of rickettsiae, there were very few in smears from treated eggs which were infected with Brazilian and Colombian spotted fevers. However, there were many rickettsiae even after paba-treatment in eggs infected with South African tick fever and fièvre boutonneuse, and, in fact, the number of rickettsiae often approached that seen in the controls. When the data in Table I are expressed graphically in terms of the cumulative percent of embryos dead per day, it is found that there has actually been more depression of the growth curve with the Rocky Mountain, Bra-

TABLE III.
Influence of Paba on Time of Death of Chick Embryos Infected with "Q" Fever, Tsutsugamushi, Lymphogranuloma Venereum, and Psittacosis.

Organism		Total No. of eggs		No. of daily deaths										% dead	Avg day of death
			Day:	2	3	4	5	6	7	8	9	10	11		
"Q" Fever	Controls	51		0	2	22	24	3	—	—	—	—	—	100	4.5
	Paba—2 mg	41		0	3	19	14	4	1	—	—	—	—	100	4.5
Tsutsugamushi	Controls	57		0	3	0	1	2	7	14	21	9	—	100	8.2
	Paba—2 mg	57		0	1	0	2	5	12	10	18	6	3	100	8.1
Lymphogranuloma venereum	Controls	32		2	14	3	2	9	2	—	—	—	—	100	4.3
	Paba—2 mg	32		1	13	4	4	5	5	—	—	—	—	100	4.4
Psittacosis	Controls	12		0	9	3	—	—	—	—	—	—	—	100	3.25
	Paba—4 mg	11		0	9	2	—	—	—	—	—	—	—	100	3.18

zilian, and Colombian spotted fevers than with either South African tick fever or fièvre boutonneuse. Figs. 1 and 2 illustrate the two extremes in reactivity to paba. A typical S-shaped growth curve is obtained in every case, but with paba-treatment the curve is flattened in varying amounts according to the kind of rickettsiae tested. The fact that South African tick fever had more survivors in the paba group, in spite of the lesser depression of its growth curve and the lesser inhibition of rickettsial growth, is explained by the lower toxicity of this kind of rickettsia for the chick embryo. The lower toxicity of South African tick fever rickettsiae is evident in the longer survival time of control embryos (average day of death = 5.1) than with any other member of the spotted fever group. Thus, even a small inhibition is sufficient to permit more embryos to survive to hatching. Fièvre boutonneuse rickettsiae are even less inhibited by paba (Fig. 2). This is of interest in view of the fact that Plotz, Reagan, and Wertman[5] could differentiate between Rocky Mountain spotted fever and fièvre boutonneuse by means of the complement fixation test. Other data of these same authors (to be published) indicate that there are wider antigenic differences between the African group and the American group of spotted fevers than between individual members of each group. This segregation in the spotted fever group is confirmed by our data on chemotherapy.

Organisms Not Responding to Maximal Dosages of Paba: In contrast to the striking inhibition of the growth of typhus[1] and spotted fever rickettsiae, it was found that the rickettsiae of "Q" fever and tsutsugamushi were apparently unaffected even by dosages of 2-4 mg of paba per egg. The data are presented in Table III. There was no significant difference between the controls and the embryos receiving paba, either in the average day of death or in the number of rickettsiae seen in smears from the yolk sacs.

Similarly, it was found that maximal amounts of paba had no apparent effect on the growth of elementary bodies of lymphogranuloma venereum and psittacosis (Table III).

Mode of Action of Paba: In a preceding report[1] we presented data showing that the acetyl derivative of p-aminobenzoic acid and the ortho- and meta- isomers of aminobenzoic acid were inactive on rickettsiae. It was clear from these results that the amino group could not be altered, and further that the para position was necessary in order for the aminobenzoic acid molecule to retain its inhibitory activity.

We then set out to test other compounds which were benzene ring analogs of paba, but with various modifications of the carboxyl group.[†] When it was found that a compound was not sufficiently soluble in water so that

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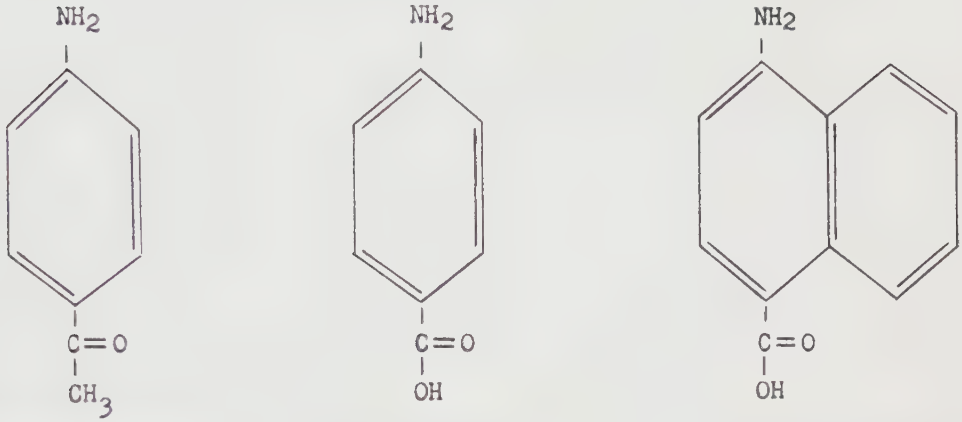
[5] Plotz, H., Reagan, R. L., and Wertman, K., PROC. SOC. EXP. BIOL. AND MED., 1944, **55**, 173.

[†] The author is indebted to Major D. A. MacFadyen, M.C., of the Army Medical School, for suggesting the use of several of these compounds, to Col. H. Plotz, M.C., of the U. S. A. Typhus Commission, for procuring them, and to Dr. W. H. Engels, of the Merck Research Laboratory, for making them available for our use.



p-aminoacetophenone p-aminobenzoic acid 4-amino-1-naphthoic acid

Fig. 3.

equimolecular amounts could be tested in comparison with paba, then both the paba and the compound to be tested were dissolved in sterile olive oil. It was found that chick embryos were relatively unaffected by injections of as much as 0.4 cc in the yolk sac, and the oil did not diminish the activity of paba.

The first compounds to be considered are those in which the carboxyl group is removed from its attachment to the benzene ring. Such a compound is p-aminophenylacetic acid ($NH_2 \cdot C_6H_5 \cdot CH_2 \cdot COOH$) in which the carboxyl group is separated from the ring by a single CH_2 group, but otherwise maintains its para position relative to the amino group. This compound was inactive on rickettsiae, even in amounts as large as 2 mg per egg. Other similar compounds which confirmed this result were p-aminocinnamic acid ($NH_2 \cdot C_6H_5 \cdot CH = CH \cdot COOH$) and p-aminobenzoyl-d, l-glutamic acid in which the chain is progressively longer which separates the carboxyl group from its ring position. Neither compound was effective in saturated aqueous solution, even though all three of these compounds have been reported by others to have effects similar to those of paba on the growth of bacteria.[6,7,8]

Other compounds were tested, in which the carboxyl group was replaced by other kinds of carbonyl groups. An example of this type is p-aminoacetophenone (Fig. 3). This compound proved toxic for the chick embryo in high concentrations (2 mg/egg), but as much as 0.5 mg could be tolerated, which in terms of an equimolecular amount of paba is well above the threshold of paba-activity. No activity was observed on the time of death of infected embryos in the presence of p-aminoacetophenone, and fully as many rickettsiae were found in smears from dead eggs as in the controls. Confirmatory evidence was obtained by using 4,4′-diaminobenzophenone, which also proved to be inactive.

Since it was now clear that the integrity and relative positions of both the carboxyl and amino groups must be maintained in order to retain activity, we proceeded to test the effect of modifying the shape or size of the molecule. This can be done by the addition of extra side chains at other points on the benzene ring, without touching the 1- and 4- positions occupied by the amino and carboxyl groups. Two such compounds, 2-acetylamino-4-aminobenzoic acid, and 3-bromo-4-aminobenzoic acid, proved to be inactive. Another way of enlarging the molecule is to add an additional benzene ring so as to form a naphthalene nucleus. The compound thus formed, 4-amino-1-naphthoic acid, is homologous to p-aminobenzoic acid (Fig. 3). Equimolecular

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[6] Rubbo, S. D., and Gillespie, J. M., *Lancet*, 1942, **242**, 36.

[7] Johnson, O. H., Green, D. E., and Pauli, R., *J. Biol. Chem.*, 1944, **153**, 37.

[8] Auhagen, E., *Hoppe-Seyler's Z. f. physiol. Chem.*, 1942, **277**, 197.

amounts of 4-amino-1-naphthoic acid were toxic for the chick embryo even at the level of the minimal effectual dosage, but some of the embryos survived sufficiently long at levels well above the minimal dosage so that they could be examined for rickettsiae. For example, when a 0.5 mg dosage of paba was used (minimal effectual dosage = 0.25 mg), an equimolecular amount of 4-amino-1-naphthoic acid killed all of the embryos within 2-4 days. But smears from these embryos contained as many rickettsiae as the controls, whereas no rickettsiae were seen in smears from paba-treated embryos of the same age. It seems clear, therefore, that 4-amino-1-naphthoic acid does not appreciably affect the growth of rickettsiae.

These data all indicate that practically any possible modification of the paba molecule will diminish, and possibly even destroy, its inhibitory activity on rickettsiae. Such a high degree of specificity is usually associated with activity on an enzyme system, and leads one to speculate that the blocking of such a system by paba may be responsible for the inhibition of rickettsial growth.

Summary. 1. The maximal dosage of p-aminobenzoic acid for the chick embryo (2 mg) is effective in inhibiting the growth of rickettsiae of the spotted fever group in the following order of magnitude: Rocky Mountain s. f. > Brazilian s. f. > Colombian s. f. > South African tick f. > fièvre boutonneuse. There was much more inhibition of the American group than of the African spotted fevers.

2. p-Aminobenzoic acid was about twice as effective on Rocky Mountain spotted fever infection (46% survivors) as on either epidemic or murine typhus (20% and 26% survivors, respectively).

3. p-Aminobenzoic acid did not appreciably inhibit the growth of the agents of "Q" fever, tsutsugamushi, lymphogranuloma venereum, or psittacosis.

4. Studies on the mode of action of p-aminobenzoic acid indicate that not only must the amino and carboxyl groups remain intact and in the para position with respect to one another, but the total size of the molecule must not be altered, if the inhibitory properties are to be retained. Such a high degree of specificity suggests that paba may be acting on an enzyme system.

15039

Synaptic Transmission of the Muscles in Arthritis.*

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Thomsen, Altamirano and Luco[1] studied some aspects of the mechanism of synaptic transmission in muscles whose tendons had been cut, thus approaching the general problem of a possible synaptic perturbation by changes in the effector.

Lippmann and Selig[2] gave experimental proof to the clinical observation that muscles of diseased joints undergo a process of atrophy. These results suggested the idea that an experimentally produced arthritis might also cause changes in the neuromuscular transmission. In this paper we report some experiments that were carried out with that idea in mind.

Procedure and Methods. The experiments were performed on cats. The arthritis was produced by injecting about 0.1 cc of 3%, 5%, or 10% silver nitrate under anesthesia by ether or nembutal. The tibio-tarsal joint was

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* Aided by a grant from the National Foundation for Infantile Paralysis, Inc.

[1] Thomsen, P., Altamirano, M., and Luco, J. V., *Medicina* (Buenos Aires), 1942, **3**, 67.

[2] Lippmann, R. K., and Selig, S., *Surg. Gynec. Obstet.*, 1928, **47**, 512.

selected in all experiments, and the silver nitrate was injected into the articular cavity through the skin.

The study of the synaptic conditions was performed with the soleus and tibialis anticus muscles from 3 to 17 days following the onset of arthritis, by anesthetizing the cats intraperitoneally with nembutal (Abbott) in a dose of 0.033 g per kg of body weight dissolved in 1 cc of 25% urethane. A cannula was inserted in the trachea in order to apply artificial respiration when necessary. For the recording of muscular contraction the tibia was fixed by drills, and the tendon was attached to the short arm of a lever pulling on rubber bands. The sciatic nerve was isolated, sectioned, and silver electrodes were applied; it was stimulated by condenser discharges controlled by electronic valves. Curare (Merck) and Prostigmine (Roche) were injected intravenously.

Results. The injection of silver nitrate into the joint caused in every case articular lesions of different intensity, ranging from congestion of the articular cartilage to necrosis and caries of the bone. Sometimes, and especially when using 10% silver nitrate, the inflammatory process caused a rupture of the capsule and a spread to peri-articular areas. These lesions resulted in an abnormal gait from immobilization of the tibio-tarsal joint, and the animals would not use their inflamed limb or would use it discreetly. This spontaneous immobilization had a tendency towards flexion.

Both the soleus and tibialis anticus on the side of the arthritis, showed a definite loss of weight when compared with their homologues of the opposite leg.

Synaptic transmission in these muscles was studied by indirect stimulation at various frequencies, by controlling the post-tetanic potentiation and by observing the action of some drugs with synaptic effect.

A. Stimulation at Various Frequencies. Rosenblueth and Cannon[3] found that the indirect stimulation of a muscle at various frequencies produces a series of rises and falls in tension called stages 1, 2, 3a, 3b, and 3c. The study of these stages in our experimental animals showed that, in 29 of 34 cases the stages 3a and 3c were more marked in the muscles of the arthritic side than in those of the normal side. Fig. 1 and 2.

B. Post-tetanic Effects. In all the experiments in which we studied this effect, we found that it was present with practically the same intensity on the muscles of both sides.

C. Curare. The action of curare was studied at low frequencies. In this way it was easier to detect differences in the muscles. In

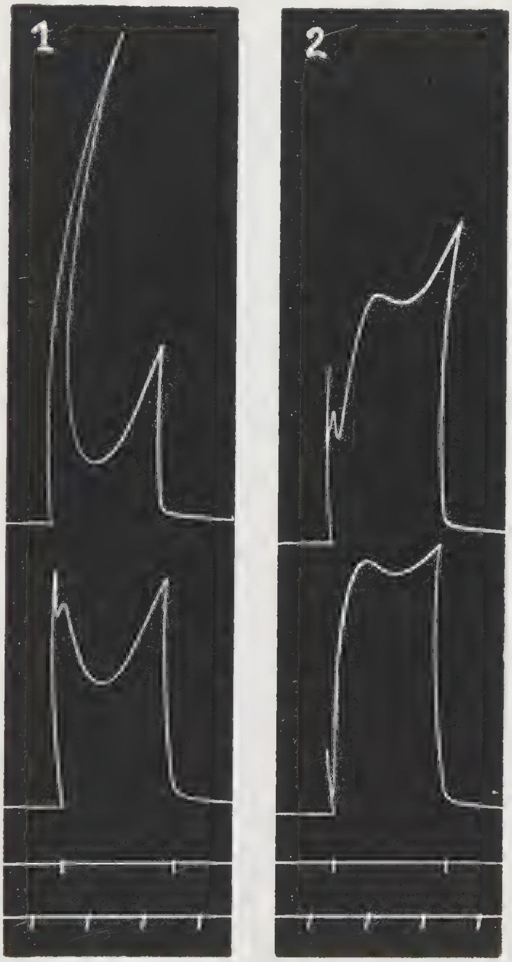

FIG. 1 AND 2.
Arthritis of 11 days (5% silver nitrate).
Upper tracing: normal soleus.
Lower tracing: arthritic soleus.
Upper marks: stimulation.
Lower marks: time in minutes.
Fig. 1. Indirect stimulation at 200 per sec.
Fig. 2. Indirect stimulation at 350 per sec.

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[3] Rosenblueth, A., and Cannon, W. B., *Am. J. Physiol.*, 1939, **128**, 31.

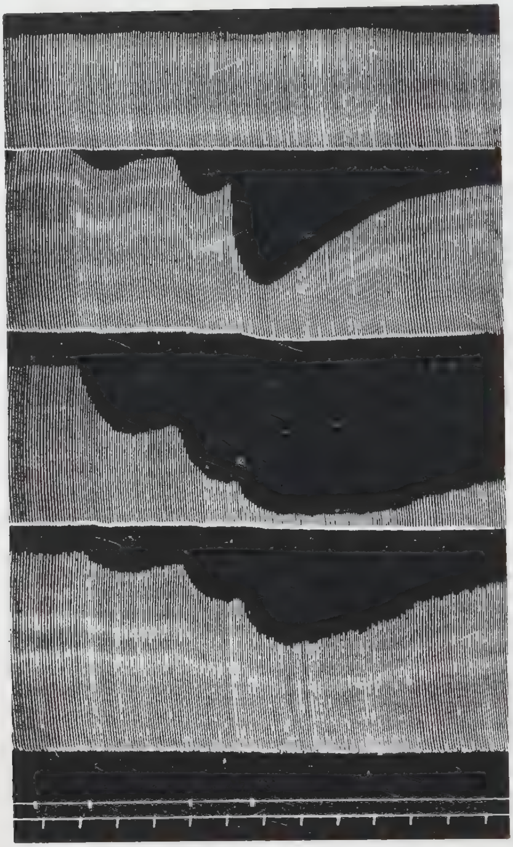

FIG. 3.
Effect of Curare on Arthritic Muscles.
Arthritis of 13 days (5% silver nitrate).
From the top the tracings are: arthritic tibialis, normal tibialis, normal soleus, and arthritic soleus.
Upper marks: doses of curare.
Lower marks: time in min.

nine experiments performed we found that the atrophic muscles were much more resistant to the effects of curare than were those of the normal limb. Fig. 3.

D. Prostigmine. In this series we also used stimuli of low frequencies. This drug produced a greater potentiating effect in the normal and a greater depressing effect in the muscles of the arthritic limb.

Discussion. The results reported here about the behavior of the synapsis under indirect stimulations at various frequencies, show that there is a difference between the normal muscle and the arthritic muscle, and that there are similarities between the arthritic muscle and the tenotomized and immobilized muscles. In the last 3 conditions, stages 3a and 3c are greater, there is a larger development of tension than in the normal when compared with stage 1. Normal, fatigued muscle (Thomsen and Luco[4]) responds similarly to the above ones.

Thomsen and Luco[4] have attributed, hypothetically, the synaptic changes in tenotomized and immobilized muscles to an exaggeration of the postural reflexes that would bring about a state of synaptic fatigue and, therefore, a state similar to the fourth experimental stage.

In the case of arthritis there is a spontaneous immobilization of muscles from pain and the same hypothesis is applicable. This reasoning would explain the similar results obtained in all these cases with stimulations at various frequencies. Besides, the resistance to the effects of curare is more marked in tenotomized, immobilized, arthritic, and fatigued muscles, than in normal ones.

In regard to prostigmine, both the arthritic and tenotomized muscles (Luco and Thomsen[5]) show that, with the same dosage and frequency, the potentiating effects are more marked in the normal and the depressive effects more marked in the altered muscles. This has been explained in the tenotomized muscle as due to its greater sensibility to acetylcholine, in such a way that prostigmine brings about a paralytic level rather quickly (Rosenblueth and Morison[6]). This would also explain the greater resistance to the effects of curare of the muscles in all the conditions mentioned above.

As a result of exaggeration of reflexes or some other cause, there could be a common denominator that would explain the similarity of the synapsis in the normal muscle fatigued by intense stimulation, and in the tenotomized, immobilized, and arthritic muscles. It could be that this common factor is a functional alteration, chemical or physico-chemical, of the excitable mechanism of the effector.

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[4] Thomsen, P., and Luco, J. V., *J. Neurophysiol.*, 1944, **7**, 245.

[5] Luco, J. V., and Thomsen, P., in press.

[6] Rosenblueth, A., and Morison, R. S., *Am. J. Physiol.*, 1937, **119**, 236.

In support of this hypothesis we have the results of Eccles and O'Connor[7] who have localized the effects of curare and eserine in relation with the action potentials of the terminal plate. As these potentials may not be entirely localized to the histological terminal plate (Feng[8]), it would not be surprising that a chemical or physico-chemical change of the effector might modify the effect of these drugs.

The fact that the arthritic muscle shows a post-tetanic effect and the tenotomized muscle usually does not, may mean that in the latter the synaptic alteration is quantitatively greater.

Summary. We have studied in cats the effect of an atrophy of the soleus and tibialis anticus, caused by an arthritis of the tibiotarsal joint, on some aspects of the synaptic transmission in those muscles.

1. The third stage of the neuromuscular transmission is different in the normal and arthritic muscles. Section A, fig. 1 and 2.
2. The atrophic muscle is more resistant to curarization. Section C, fig. 3.
3. Prostigmine, with the same dosage and frequency of stimulation, produces less potentiation and more depression in the arthritic than in normal muscles.
4. The synaptic behavior of the arthritic muscle is similar to the behavior of the tenotomized, immobilized, and fatigued (4th stage) muscles. The possible significance of such similarity is discussed.

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[7] Eccles, J. C., and O'Connor, W. C., *J. Physiol.*, 1939, **77**, 44.

[8] Feng, T. P., *Biol. Symposia*, The Jaques Cattell Press, Lancaster, Pa., 1941, **3**, 121.

15040

Billroth I, Gastric Resection: Extent Necessary to Protect Against Histamine-Provoked Ulcer.*

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It has been shown in this clinic that a short afferent duodenal loop is an important component of a satisfactory operation when gastric resection on the Billroth II plan of operation is applied to ulcer.[1] It has been shown also that another criterion of a successful operation for ulcer is an extensive gastric resection (75%).[2,3] Inasmuch as the short afferent duodenojejunal loop is so important, the question arose whether it would be equally as satisfactory to sacrifice less stomach (25 or 50%), but to effect gastrointestinal continuity by end to end suture between the stomach and the duodenum by the Billroth I operation. It is the purpose of this report to indicate our observations and experiences with this plan of procedure on the dog, and to note whether the Billroth I plan of performing gastric resection will protect against the histamine-in-beeswax provoked ulcer with excision of less stomach tissue than in the Billroth II operation.

Method. These experiments were carried

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* Supported by special grants for surgical research from the following sources: Citizen's Aid Society, Augustus L. Searle, Dr. and Mrs. Harry B. Zimmerman, the Dr. Berenice Moriarity, the Robert A. Cooper Funds, and a grant from the Graduate School of the University of Minnesota.

[1] Merendino, K. A., Lannin, B. C., Kolouch, F., Jr., Baronofsky, I., Litow, S. S., and Wangensteen, O. H., PROC. SOC. EXP. BIOL. AND MED., 1945, **58**, 226.

[2] Wangensteen, O. H., and Lannin, B. G., *Arch. Surg.*, 1942, **44**, 489.

[3] Lannin, B. G., Hay, L. J., Judd, E. S., Jr., and Wangensteen, O. H., PROC. SOC. EXP. BIOL. AND MED., 1944, **56**, 231.

out on 12 dogs in 3 series. Each series had a different amount of stomach resected, but the residual gastric pouch in each dog in all series was anastomosed to the duodenum just beyond the inverted duodenal end by means of an end-to-side gastroduodenostomy. This procedure, known as the Billroth I-Von Haberer plan of operation, is technically more feasible in the dog than the straightforward Billroth I operation which requires an end-to-end gastroduodenostomy. After an interval averaging 46 days, the administration of 30 mg of the histamine-in-beeswax mixture, prepared after the method of Code and Varco,[4] was injected intramuscularly each evening, following which the dogs' feed pans were removed and no more food given until the following morning. Unless the dogs succumbed from the complications of ulcer invoked by the histamine implantation, the injections were carried out for 45 days.

Operative Procedure. The dogs were anesthetized with intravenous nembutal, 15 mg per pound. Anesthesia was continued with intratracheal ether. Continuous gastric suction was applied during and immediately after surgery. Under aseptic conditions, a midline incision was made. The duodenum was transected and inverted immediately distal to the pyloric sphincter. The required amount of stomach was resected, the Hofmeister procedure being used on the lesser curvature side. Continuity of the intestinal tract was completed by an end-to-side aseptic gastroduodenostomy just beyond the inverted duodenal end. The abdomen was then closed.

Post-operatively, the dogs were fed parenterally for 72 hours, with 10% glucose in saline solution containing some isotonic gelatine in saline solution. After 72 hours water was allowed, and the diet gradually increased thereafter, until in one week the regular diet of tablescraps, horsemeat, and kibbles was tolerated. This was reinforced with vitamins A, B, C, and D.

Experiments. Series I. Four dogs were used. A 25% gastric resection and gastroduodenostomy was performed at the inverted duodenal end. After a sufficient recovery period from the operation, the daily administration of the histamine-in-beeswax mixture was begun.

Series II. The identical procedure was used on 4 dogs in this series with one difference: a 50% gastric resection was carried out, followed after a suitable interval by the administration of histamine.

Series III. In this series a three-quarter gastric resection (75%) was done, followed subsequently by the administration of histamine.

Results. The incidence of stomal ulcer attending the 25, 50, and 75% gastric resection carried out in the Billroth I plan of operation, followed by the administration of histamine, is shown in Table I. In both Series I (25% gastric resection) and in Series II (50% gastric resection) stomal ulcer was observed in 3 out of the 4 dogs in each series. In Series III (75% gastric resection) stomal ulcer was not observed.

Discussion. It would appear from these experiments that just as large a gastric resection is necessary in the Billroth I plan of operation to protect against stomal ulcer, as in the Billroth II operation. Whereas a short afferent duodenojejunal loop is an essential component of a satisfactory operation for ulcer, the factor of a short afferent loop alone cannot supplant extensive gastric resection. It is in the employment of all the criteria of a satisfactory operation for ulcer as described elsewhere[2,3] that one may obtain results that will be consistently good. The Billroth I plan of operation for ulcer, with a three-quarter gastric resection (75%), is technically a more difficult procedure than a similar resection in which a short afferent duodenojejunal loop is employed in a retrocolic anastomosis made at the ligament of Treitz (Billroth II). The latter operation has been employed in this clinic in the surgical management of ulcer for several years.

Conclusions. Small gastric resections (25 and 50%) in dogs, when accompanied by no afferent loop (Billroth I), are accompanied by a high incidence of histamine invoked stomal ulcer. When a three-quarter gastric resection (75%) is done, however, stomal ulcer cannot be produced with histamine.

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[4] Code, C. F., and Varco, R. L., PROC. SOC. EXP. BIOL. AND MED., 1940, **44**, 475.

TABLE I.
Occurrence of Stomal Ulcer Accompanying Gastric Resection of Variable Sizes (25, 50, and 75%) Carried Out on the Billroth I Plan of Operation After Administration of Histamine-in-Beeswax.

Dog No.	Wt, lb	Survival, days	Results
		Series I—25% Gastric Resection.	
356	72¼	16	4 large duodenal ulcers. One perforated.
330	45	45	Small gastric ulcer. Small duodenal erosion.
331	55	45	No ulcer.
394	30	17	2 duodenal ulcers, one perforated.
		Series II—50% Gastric Resection.	
325	45	45	Large duodenal ulcer.
333	40	45	,, ,, ,,
313	34	45	,, ,, ,,
262	38	45	No ulcer.
		Series III—75% Gastric Resection.	
328	33	31	No ulcer. Pregnant. Dead of uterine volvulus.
329	44	45	No ulcer
372	42	45	,, ,,
373	55	45	,, ,,

These experiments suggest that it is unlikely that substitution of the Billroth I for the Billroth II plan of operation, in which a short afferent duodenal loop is employed, will protect against recurrent ulcer with less sacrifice of stomach. A three-quarter resection will protect against the histamine-in-beeswax provoked ulcer, whether the operation is carried out on the Billroth I or II plan of operation.

15041

Fate of Intravenously Injected Fat: Its Role in the Production of Ulcer*

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Gastric hemorrhage as a complication of fracture has been observed in patients in this clinic.[1] In a series of 52 dogs subjected to fracture of the humerus, or to a small drill hole placed through both cortices of the humerus or curettage of the bone marrow of the shaft of the humerus after placement of the drill hole, gastroduodenal pathology (erosion and/or ulcer) was demonstrated in 21% of the dogs.[2] In dogs with gastric pouches, such procedures, as well as the intravenous injection of fat, failed to evoke evidence of stimulation of gastric secretion.[3] It was observed however that ulcer could be produced by the intravenous injection of fat, when accompanied by the administration of histamine-in-beeswax.[4] It remained to be shown, however,

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* Supported by special grants for surgical research from the following sources: Citizen's Aid Society, Augustus L. Searle, Dr. and Mrs. Harry B. Zimmerman, the Dr. Berenice Moriarity, the Robert A. Cooper Funds, and a grant from the Graduate School of the University of Minnesota.

[1] Wangensteen, O. H., Merendino, K. A., and Litow, S. S., *Bull. Am. Coll. Surg.*, 1945, **30**, 58.

[2] Merendino, K. A., Litow, S. S., Armstrong, W. D., and Wangensteen, O. H., *Bull. Am. Coll. Surg.*, 1945, **30**, 58.

[3] Merendino, K. A., Litow, S. S., and Wangensteen, O. H., *Bull. Am. Coll. Surg.*, 1945, **30**, 58.

[4] Baronofsky, I., and Wangensteen, O. H., *Bull. Am. Coll. Surg.*, 1945, **30**, 59.

TABLE I.
Production of Ulcer in Rabbits with Intravenous Fat and Histamine-in-Beeswax Mixture. (30 mg of histamine-in-beeswax given intramuscularly once daily).
(Series I—Rabbits).

Rabbit No.	Size, kg	No. of injections	Amount fat I.V., cc	Results	Remarks	Path. reports			
						B	L	K	S*
1	1.58	1	1½	Perforating ulcer greater curvature	Killed 1 day after injection.	—	+	+	+
2	1.8	2	1½	Perforating ulcer greater curvature	Killed 2 days after inj.	—	+	+	+
3	1.58	1	1½	No ulcer	Dead pulmonary embolus 1 day.	+	+	+	—
6	2.1	4	2	Entire fundus eroded away	Dead in 4 days.	—	+	+	—
7	1.8	2	1½	Duodenal ulcer	2 inj. 30 mg histamine Q.O.D. x 2. Sacrificed 4 days after last inj.	—	+	+	—
8	1.58	1	1	Perforating ulcer lesser curvature	Killed 1 day after inj.	+	+	+	—
	Controls: Intravenous fat but no histamine.								
4	1.58	0	1½	No ulcer	Sacrificed 9 days.	—	+	—	—
5	1.58	0	1½	” ”	” 10 ”	—	+	—	—
9	1.58	0	1½	” ”	” 14 ”	—	—	—	—
	Controls: Histamine but no intravenous fat.								
10	1.8	28	0	No ulcer	” 28 ”	—	—	—	—
11	1.58	21	0	” ”	” 21 ”	—	—	—	—

* B—Brain. L—Lung. K—Kidney. S—Stomach.
— = No emboli.
+ = Fat emboli present.

whether fat can be demonstrated in the end vessels of the gastric mucosa and submucosa in animals injected with fat in which ulcer was produced by intravenous injection of fat. This study concerns itself with that item.

Method. Two series of observations were carried out on 52 animals consisting of 15 rabbits, 18 dogs, 13 cats and 6 guinea pigs. A single, intravenous injection of fat was made in all the animals. Human breast or omental fat obtained from surgical procedures and extracted with ether was used. One and one-half cc of fat per kg of body weight was injected intravenously in all animals except in the guinea pigs, in which the injection was made directly into the heart.

The observations on these 52 animals are divided into 2 series. In the first, consisting of 26 animals, the presence or absence of ulcer attending the intravenous injection of fat was noted. It should be mentioned here that only 6 rabbits among the 52 animals employed in these experiments received, in addition to the intravenous fat, a daily injection of 30 mg of histamine-in-beeswax, prepared after the method described by Code and Varco.[5]

A second series of observations was then carried out on the fate of the intravenous fat, as studied by microscopic section in the 24 of the 26 animals in Series I that received fat intravenously. Twenty-eight additional similar observations were made on 7 cats, 11 dogs, 4 guinea pigs and 6 rabbits.

Experiments: Series I. Eleven rabbits, 6 cats, 7 dogs, and 2 guinea pigs were used. Six of the rabbits received both the fat and the preparation of histamine-in-beeswax mixture. Three received fat alone and 2 received histamine alone. Of the 7 dogs, 5 received fat alone, and the remaining 2 had the left and right gastric and gastro-epiploic vessels ligated in addition to the fat injection. Both the cats and guinea pigs were submitted to intravenous fat only.

Series II. Fifteen rabbits, 18 dogs, 6 guinea pigs, and 13 cats were used. These were injected intravenously with about 1.5 cc of fat per kg of body weight and were sacrificed in

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[5] Code, C. F., and Varco, R. L., PROC. SOC. EXP. BIOL. AND MED., 1940, **44**, 475.

TABLE II.
Production of Ulcer in Various Animals with Intravenous Fat (No Histamine).
(Series I)

A. No. of cats	Wt	Amt of fat	Time of sacrifice	Results	Remarks
6	1 to 2 kg	1.5 to 2 cc per kg	1 to 21 days after inj. of fat	Ulcers in 2 of 6 cats. Ulcers on greater curvature of stomach of cats sacrificed 4 and 18 days after fat inj.	Both showed emboli in brain, lung, kidney. Submucosal vessels of stomach of the cat sacrificed after 18 days showed fat emboli.
B. No. of dogs					
7	8 to 10.5 kg	1.5 cc per kg	Same	One dog with bleeding duodenal ulcer 14 days after fat inj.	This ulcer occurred in one of 2 dogs that had major arteries and veins of stomach, except the short gastric branches of splenic vessels ligated.
C. No. of guinea pigs					
2	600 and 800 g	1 cc	2 and 4 days after inj. of fat	Typical gastric ulcers in the guinea pig sacrificed 2 days after fat inj.	Emboli in brain, kidney, and lung. Emboli in stomach near ulcer.

TABLE III.
Occurrence of Fat Emboli Attending the Intravenous Injection of Fat (Series I and II).

	No. of animals	Amt of fat injected cc/kg	% of tissues revealing fat emboli			
			Lung	Brain	Kidney	Stomach
		Sacrificed 1 to 4 days after fat injection.				
A.	23	1½	91	60	73.9	47.8
		Sacrificed 5 to 21 days after fat injection.				
B.	29	1½	41	11.1	34.4	3.7

periods varying from one to 21 days. Sections were taken from lung, brain, kidney and stomach and stained for fat emboli.

Results. The incidence of erosion or ulcer (gastric and/or duodenal) attending the intravenous administration of fat with or without histamine is shown in Tables I, II, and III. In Series II, in animals sacrificed within one to 4 days after the intravenous injection of fat, the occurrence of fat embolism in the various tissues was as indicated in Table III.

Discussion. These results show that fat emboli released in the blood stream may occlude the end vessels in the gastric or duodenal mucosa, causing areas of local anemia; thus these areas become susceptible to the acid-peptic digestive activity of the gastric juice. It has been shown previously in this laboratory that it is difficult, if not impossible, to produce acid peptic ulcers in the rabbit using histamine alone.[6] Yet, with a combination of histamine and fat intravenously, ulcer can be produced quite consistently. The mechanism of ulcer production undoubtedly is plugging of the end vessels to the mucosa. Even though intravenously injected fat does not stimulate nor augment gastric secretion, the resultant anemic areas in the mucosa become less resistant to injury and digestion by the acid-peptic juice than is the normal mucosa. Microscopic studies of the gastric wall of animals into which fat has been injected intravenously indicate definitely that the rate of disappearance of the fat from the mucosal and submucosal vessels is rapid. This circumstance undoubtedly accounts for the fact that ulcer or hematemesis have not been observed more commonly to accompany fracture of long bones in man. That fat emboli in the lung and brain are common occurrences in patients dying early after fracture of long bones is well known. In response to an inquiry addressed to 50 American orthopedic surgeons concerning the occurrence of hematemesis or ulcer after fracture, 42 replies were received. None reported observing ulcer or bleeding after fracture. However, two surgeons each reported having observed hematemesis once after the manipulation of a stiff joint under anesthesia. It has been indicated previously that the production of chronic arterial spasm also invites the occurrence of erosions and ulcer.[8] In other words, even temporary areas of anemia in the gastric or duodenal wall render those areas prone to injury by the gastric juice. Arterial thrombosis of gastric vessels, as well as arteriosclerosis of the terminal gastric vessels probably abet the ulcer diathesis in patients whose stomachs secrete normally. It would appear to be justifiable to suggest that bacterial emboli, lodging in the end vessels of the gastric mucosa, may also invite erosions and hematemesis

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[6] Hay, L. J., Varco, R. L., Code, C. F., and Wangensteen, O. H., *Surg., Gyn. and Obst.*, 1942, **75**, 170.

Conclusions. Fat emboli may cause gastric and/or duodenal erosions or ulcers. The presence of fat emboli after intravenous injection of fat may be demonstrated in the lung, brain, kidney and stomach. Its rate of disappearance in experimental animals is such that after 4 days it is found in sections of lung tissue in 41%, brain 11.1%, kidney 34.4% and the stomach of 3.7% of the sections examined. On the contrary, in sections of those animals sacrificed within one to four days after the intravenous injection of fat, the incidence of demonstrable fat emboli in the same tissues was: lung 91%, brain 61%, kidney 73.9% and stomach 47.8%.

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[7] Response to an inquiry addressed to 50 American orthopedic surgeons.

[8] Baronofsky, I., and Wangensteen, O. H., *Bull. Am. Coll. Surg.*, 1945, **30**, 59.

15042

Obstruction of Splenic Vein Increases Weight of Stomach and Predisposes to Erosion or Ulcer.*

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Bleeding from esophageal varices, in conditions which give rise to portal hypertension, occurs not infrequently. The usual explanation is that such bleeding has its origin in the increased hydrostatic pressure; in other words, rupture of the mucosal and submucosal veins occurs. At autopsy or operation, unusually large veins are observed commonly in the omenta of the upper end of the stomach and about the lower end of the esophagus. Patients with obstruction of the superior vena cava, exhibiting esophageal varices, do not bleed, however. A more likely cause of bleeding, therefore, in portal hypertension would appear to be erosion by the gastric acid-peptic digestive juice. The purpose of this presentation is to test this latter thesis experimentally.

Methods. These experiments were carried out on rabbits and dogs in 3 series. In each series an obstruction to the normal venous return of part or all of the stomach to the portal system was made. In 2 of the series, the normal flow of venous blood from the left gastro-epiploic vein into the splenic was ob-

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* Supported by special grants for surgical research from the following sources: Citizen's Aid Society, Augustus L. Searle, Dr. and Mrs. Harry B. Zimmerman, the Dr. Berenice Moriarity, the Robert A. Cooper Funds, and a grant from the Graduate School of the University of Minnesota.

structed by a tie placed proximal to their juncture. In the third series cellophane was placed snugly around the portal vein as it lay in the gastrohepatic omentum. Pearse[1] has shown that cellophane, when placed around the aorta, will slowly obliterate this vessel, an occurrence occasioned through the agency of a severe fibroblastic reaction within 6 weeks of the placement of the cellophane ligature. These procedures were tolerated very well by the animals, and all animals were eating and drinking normally as soon as the effects of the anesthetic wore off. After an interval of 2 days after operation in the splenic-tie series and an average of 113 days in the portal-tie series, the administration of 30 mg of the histamine-in-beeswax mixture prepared after the method of Code and Varco[2] was injected each evening. The dogs' feed pans were removed and no more food given until the following morning. No restriction of food intake was exercised in the instance of the rabbits. The time of sacrifice in the dogs was determined by the occurrence of spontaneous hematemesis or melena or extreme weakness. The rabbits were sacrificed at varying periods of time. In all animals the stomachs were weighed. An effort has been simultaneously made to garner control data on the weights of normal stomachs in both rabbits and dogs.

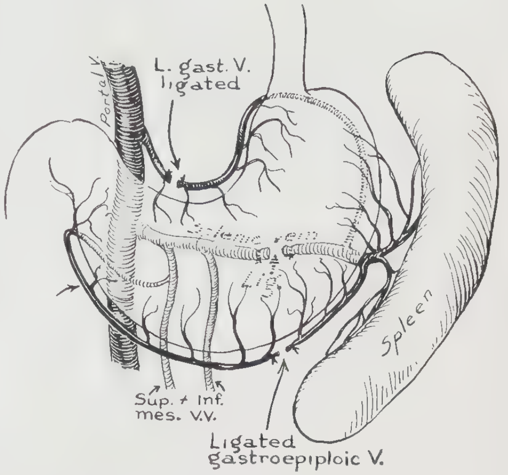

FIG. 1.

Sites of ligature of splenic, left gastro-epiploic, and left gastric vein as employed in these experiments.

Operative Procedure. The animals were anesthetized, and under aseptic conditions a laparotomy was performed. The lesser omental bursa was entered by retracting the stomach upward and to the left; an avascular area in the omentum was opened as it came off the greater curvature of the stomach. The splenic vein was identified and dissected down to its junction with the left gastro-epiploic vein. (Fig. 1.) In the dog this is quite a constant anatomical feature. Ligatures were then placed proximal to this junction and distal to where the inferior mesenteric vein entered the splenic. The splenic vein was cut between these ligatures. The left gastro-epiploic vessels were then divided and ligated about at the mid-point of the greater curvature. The spleen enlarged to three times normal upon ligating the splenic vein. An additional ligature and division was made of the left gastric vein on the lesser curvature before its entry into the portal vein.

In the series of experiments in which the portal vein was obstructed, the same approach was used. Upon identifying the portal vein and dissecting it for a distance of a centimeter or more, a piece of cellophane was placed snugly around it. This was knotted, care being observed that this procedure did not cause an immediate complete obstruction of the portal vein.

Experiments. Series I. Seven dogs were used in this series. The splenic vein was ligated and the histamine-in-beeswax mixture administration was begun 2 days after surgery. Two dogs in the series did not receive any histamine.

Series II. Four dogs were used in this series. The portal vein was obstructed as described above. To 2 of these 4 dogs, after a comparatively long interval (113 days), the administration of histamine-in-beeswax was commenced.

Series III. Fourteen rabbits were used. Ligature and division of the splenic vein was performed, and followed by histamine-in-beeswax administration to 9 of the 14 rabbits.

Results. The dogs with splenic-tie deteri-

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[1] Pearse, H. E., *Ann. Surg.*, 1940, **112**, 923.

[2] Hay, L. J., Varco, R. L., Code, C. F., and Wangensteen, O. H., *Surg., Gyn. and Obst.*, 1942, **75**, 170.

TABLE I.
Production of Ulcer in Dog with Splenic-tie Followed by Administration of Histamine-in-Beeswax Mixture.

Dog No.	Wt in lb	Daily dose of histamine base, mg	No. of inj.	Results	Sacrificed after splenic-tie, days	Wt stomach, g
367	30	30	4	Large duodenal ulcer. Bleeding points and erosion in esophagus	6	210
368	44	30	2	2 large bleeding ulcers. Tremendous amt of blood in stomach	4	220
374	28	30	2	Large duodenal ulcer. 2 esophageal erosions	4	178
375	36	30	2	3 large duodenal ulcers. Esophageal erosions.	4	201.5
382	25	30	2	Stomach filled with fresh blood and clots. Fundus mucosa desquamated and submucosal vessels bare.	4	193.2
Controls: (Splenic-tie but no histamine)						
362	28	0	0	No ulcers	Sacrificed 71 days after tie.	192
363	30	0	0	” ”	Same	201
Controls: (Histamine-in-beeswax without splenic-tie)						
1	26	30	2	No ulcers or erosions.	Sacrificed 2 days after injection started.	135.5
2	37	30	3	” ” ” ”	Same	131.3
3	26	30	4	” ” ” ”	Same	125

TABLE II.
Production of Ulcer in Dogs with Portal Vein Obstruction and Histamine-in-Beeswax Mixture.

Dog No.	Wt in lb	Daily dose of histamine base, mg	No. of inj.	Results	Remarks	Wt stomach, g
317	14.5	30	2	Large duodenal ulcer. Bleeding esophageal varix.	Sacrificed 2 days after injections started.	157.5
318	15	30	3	Perforating duodenal ulcer. Multiple ulcers in stomach.	Same, 3 days after	135
Controls: (Portal vein occlusion. No histamine)						
319	26	0	0	No ulcer.	Sacrificed 101 days after operation	201.5
278	27	0	0	No ulcer. Old submucosal hemorrhages in esophagus	Same, 150 days after	208

orated rapidly after the commencement of histamine administration. The average number of injections necessary to produce very large ulcers in all animals was 2.6. The results are tabulated in Table I.

The results of Series II (cellophane) are tabulated in Table II. The results parallel closely those of Series I. Examination of the scatter graph in Fig. 2 indicates definitely that the dogs with splenic or portal vein obstruction have much heavier stomachs.

There was no consistent enlargement or fibrosis of the spleens, although in one of the control splenic-tie dogs the spleen was covered by a thick plaque of fibrous tissue, though the spleen itself was not abnormal in size. The collateral circulation in the portal obstruction series was beautifully demonstrated. The veins of Sappey, the veins of Retzius, the superior hemorrhoidal vein anastamoses, the esophageal veins, and the veins coursing through the omentum and joining the anterior abdominal wall veins were enlarged and prominent uniformly. There was minimal ascites

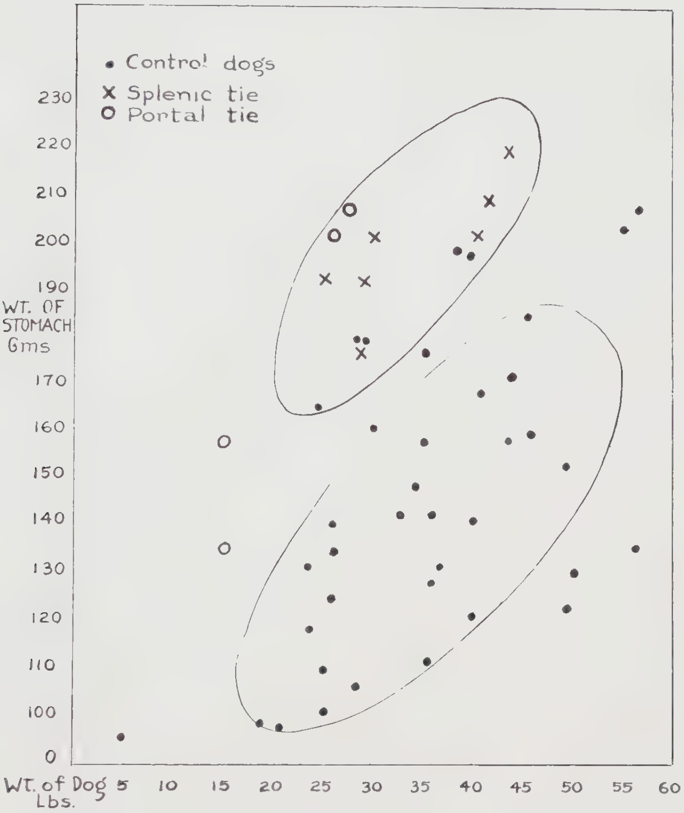

FIG. 2.

Scatter graph showing heavier weights of the stomachs of dogs with splenic vein-ties and portal vein obstruction.

in one of the dogs with portal obstruction. In the 4 dogs with occlusion of the portal vein, all save one at autopsy presented complete obstruction of the vein; in that one dog, there was a small residual lumen detected only on microscopic examination.

Ulcers and/or erosions were produced in 7 out of 9 rabbits with splenic-tie followed by histamine administration. (Table III.) The stomach weights for this series averaged 32.03 g as compared with 21.4 g for the control series of normal rabbits (Table IV).

Comment. It is apparent from these experiments that obstruction of the venous drainage from the stomach abets the ulcer diathesis. That is, erosions and ulcer are far more readily provoked with histamine in the presence of portal hypertension than when there is no obstruction to venous outflow of blood from the stomach. We have been unable in the rabbit to produce ulcer with histamine alone.[2] However, as is indicated herein, bleeding ulcers and erosions follow regularly when histamine administration is preceded by ligature and division of the splenic vein. Moreover, as was indicated elsewhere,[3] perforated ulcers may be brought about quite regularly in the rabbit by the production of chronic arterial spasm achieved by the implantation of epinephrine in beeswax, when accompanied by the simultaneous administration of histamine.

In the dog, too, ulcer is produced regularly in a surprisingly short time when the venous drainage from the stomach is obstructed, accompanied by the administration of histamine. Esophageal varices were observed regularly in the experiments of longer duration, in which the portal vein was obstructed. Esophageal erosions were observed in several of the dogs.

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[3] Baronofsky, I., and Wangensteen, O. H., *Bull. Am. Coll. Surg.*, 1945, **30**, 59.

TABLE III.
Production of Ulcer in Rabbit with Splenic Vein-Tie Followed by the Administration of Histamine-in-Beeswax.

Rabbit	Size	Daily dose of histamine base, mg	No. of inj.	Results	Remarks
1	Adult	30	1	Erosions and bleeding ulcers.	Sacrificed.
2	,,			Linear erosions of apices of rugæ.	Animal fasted for 2 days pre-operatively.
3	,,	30	2	Erosion and bleeding ulcers.	Sacrificed.
4	,,	30	1	Free blood in stomach. Enlarged varix at esophageal junction. Bleeding ulcers and erosions.	,,
5	,,	30	2	Bleeding ulcers and erosions.	,,
6	,,	30	7	Bleeding erosions at cardia.	,,
7	,,	30	7	No ulcers.	,,
8	,,	30	2	Bleeding erosions and ulcers.	,,
9	,,			No ulcers.	,, 25 dys.
10	,,	30	17	Bleeding erosions.	,,
Controls: (Splenic-tie without histamine)					
					No histamine. Sacrificed 17 days.
11	Adult	0	0	No ulcers or erosions.	Same, 12 days.
12	,,	0	0	,, ,, ,, ,,	Same, 12 days.
13	,,	0	0	,, ,, ,, ,,	Same, 8 days.
14	,,	0	0	,, ,, ,, ,,	No splenic-tie.
Controls: (Histamine-in-beeswax without splenic-tie)					
15	Adult	30	17	No erosions or ulcers.	No splenic-tie, Died of histamine shock.
16	,,	30	21	,, ,, ,, ,,	
17	,,	30	21	,, ,, ,, ,,	No splenic-tie.
18	,,	30	28	,, ,, ,, ,,	,, ,, ,,

TABLE IV.
Weights of Stomachs of Rabbits Subjected to Splenic Vein Ligation With or Without Histamine.

No. of rabbits	Procedure	Avg wt of rabbits, kg	Avg wt of stomachs in g
13	Splenic-tie, histamine	1.8	32.03
11	No splenic-tie, no histamine	1.8	21.4
Controls:			
4	Histamine-in-beeswax 30 mg every day for 17, 21, 21, 28 days resp. No splenic-tie.	1.8	21.3

Another striking finding was the uniform increase in weight of the stomachs of both rabbits and dogs in which obstruction to the venous outflow from the stomach had been established. Microscopically, this occurrence appears to be due to an edema of the entire gastric wall, but especially of the mucosa and submucosa. Erosion of the mucosa by acid occurs readily when the blood supply has been altered by obstructing the venous outflow.

Experience with patients with occult bleeding from the alimentary tract suggests, too, that acid-peptic erosion is a frequent source of the bleeding. In this clinic we have come slowly, but definitely, to this conclusion. During the past few months one of us (O. H. W.) has submitted two patients with portal hypertension and severe anemia from repeated hematemesis (one with, the other without cirrhosis) to extensive gastric resection (90%) with cessation of the bleeding and very gratifying results.

Conclusions. 1. Partial obstruction to the venous outflow from the stomach increases its weight in rabbits and dogs. 2. Esophageal varices can be produced by portal or splenic vein obstruction and, when histamine-in-beeswax is given to such animals, esophageal and gastric erosions and bleeding are produced. 3. Portal or splenic vein obstruction abets the ulcer diathesis.

15043

Augmentation of Sheep Pituitary Gonadotrophin by Insoluble Metallic Hydroxides.*

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Urine extract, blood serum, yeast and yeast ash, zinc and copper salts have been shown to increase the gonadotrophic activity of pituitary gland preparations.[1-8] In addition formed elements of blood[9] and heme prepared from erythrocytes augment the gonadotrophic action of pituitary preparations,[10] but not the gonadotrophin in pregnant mare's serum or that in the urine of pregnant women.[11]

As part of a more extensive investigation directed toward finding methods for increasing the gonadotrophic activity of pituitary preparations, a study was made of the effect of aluminum, magnesium, iron and zinc hydroxides on increasing the effectiveness of pituitary gonadotrophic substances. The results obtained from this investigation provide the substance of this report.

Experimental methods: 1. Gonadotrophic extracts. Relatively crude and purified sheep pituitary extracts, a follicle-stimulating preparation, and an extract of rat pituitary glands were used in this study. The cruder of the two unpurified extracts was obtained by extracting sheep pituitary powder with water in the ratio of 1 gm powder to 10 ml water. The extraction was repeated once, the 2 extracts were combined, and the active substance was precipitated from this aqueous extract by the addition of 4.5 volumes of acetone. The precipitate was removed from the acetone by decanting and centrifuging. This insoluble material was extracted with water after which the active substance was precipitated with acetone and dried by washing with acetone. The other extract of this type was the pH 5 soluble, B fraction, previously reported.[12]

The purified preparation which was used with aluminum hydroxide was of low solid content and contained the major part of the activity of the original pituitary powder.[12] The follicle stimulating preparation was obtained by tryptic digestion.[13] An aqueous extract of air dried rat pituitary glands was made and lyophilized, and this preparation was tested with aluminum hydroxide.

2. The preparation of hydroxides. The aluminum hydroxide was prepared essentially by the method reported by Johnson and Bradley.[14] Two hundred seventy grams of ammonium alum that contained 12 molecules of water of crystallization were dissolved in 1 liter of distilled water by heating. Seventy-five milliliters of concentrated ammonium hydroxide were diluted to 2 liters with distilled water. The alum solution was added slowly to the ammonium hydroxide while a stream of

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* Supported in part by a grant from the Wisconsin Alumni Research Foundation.

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[2] Cole, H. H., and Hart, G. H., Proc. Soc. Exp. Biol. and Med., 1934, **32**, 370.

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[5] Hellbaum, A. A., Proc. Soc. Exp. Biol. and Med., 1936, **33**, 586.

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[8] Kraatz, C. P., *Am. J. Physiol.*, 1936, **117**, 250.

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[10] McShan, W. H., and Meyer, R. K., *Am. J. Physiol.*, 1937, **119**, 574.

[11] McShan, W. H., and Meyer, R. K., *Endocrinology*, 1941, **28**, 694.

[12] McShan, W. H., and Meyer, R. K., *J. Biol. Chem.*, 1943, **151**, 259.

[13] McShan, W. H., and Meyer, R. K., *J. Biol. Chem.*, 1940, **135**, 473.

[14] Johnson, C. A., and Bradley, W. B., *J. Infect. Dis.*, 1935, **57**, 70.

TABLE I.
Augmentation of Sheep Pituitary Gonadotrophin by Aluminum Hydroxide.

No.	Gonadotrophic extract, mg*	Aluminum hydroxide, mg	No. of rats	Wt of ovaries, avg, mg
25B	100	—	3	52
,,	100	100	3	354
,,	50	—	3	25
,,	50	50	3	229
,,	25	—	3	13
,,	25	25	3	98
,,	12.5	—	3	13
,,	12.5	12.5	3	24

* Indicates mg equivalents of dry pituitary powder used in making the preparation.

steam was passed into the mixture. The steam treatment was continued for 15 minutes after the final addition of the alum or until the mixture boiled. The equivalent of the alum used was in excess of that of ammonium hydroxide.

The zinc, iron and magnesium hydroxides were prepared by the same general procedure using zinc sulphate and ferric ammonium alum with ammonium hydroxide, and magnesium sulphate with dilute sodium hydroxide. Each of the preparations was kept in the incubator at 75°C for 12 hours after which the supernatant liquid was decanted and distilled water was added while stirring. A number of decantations were made over several days until the supernatant solution was relatively free of the various salts after which the hydroxide was suspended in a definite volume of water. The amount of hydroxide required was measured from these stock suspensions, centrifuged, decanted and mixed with the extract to form a suspension.

3. Assays. The combination of the different gonadotrophic preparations and the hydroxides were made by mixing *in vitro* a given quantity of the gonadotrophin with a definite amount of the various hydroxides. The gonadotrophic preparations were tested alone in the same quantities as was used with the hydroxides. The proper amounts of these preparations were injected subcutaneously into 21-day-old female rats. The rats were injected with 0.5 ml during the afternoon of the first day, and twice daily with this amount on each of the 4 succeeding days. The animals were killed during the morning of the sixth day, the ovaries were removed, examined before a strong light for the purpose of determining the presence of, and relative numbers of follicles and corpora lutea, and weighed. The increase in the weight of the ovaries obtained with the hydroxide-gonadotrophic complex over that obtained with the extract alone was used as a measure of the amount of augmentation obtained.

Results and Discussion: The data presented in Tables I and II are a summary of the results obtained with aluminum, iron, zinc and magnesium hydroxides. The crude extract, 25B, obtained by the acetone precipitation method was tested using decreasing amounts of the extract with decreasing amounts of the aluminum hydroxide. As the data show there was a corresponding decrease in the activity under these conditions. When 100 mg[†] of the extract was given with 100 mg of aluminum hydroxide the ovaries obtained had an average weight of 354 mg. Intermediate values were obtained when 50 and 25 mg of the gonadotrophin and the hydroxide were given. The smallest amount of each of the substances, 12.5 mg, produced ovaries which had an average weight of 24 mg. It is of interest to note that 12.5 mg and 25 mg of the gonadotrophin alone did not stimulate the ovaries.

The other relatively crude extract that was used was 28B. Fifty milligrams of this extract was given with varying amounts of aluminum hydroxide and the data are

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[†] Indicates in all cases mg equivalents of pituitary powder used as beginning material in making the various preparations.

TABLE II.
Augmentation of Pituitary Gonadotrophins by Insoluble Metallic Hydroxides.

Kind	Gonadotrophic preparation, amt, mg*	Augmenter, $Al(OH)_3$ mg	No. of rats	Wt of ovaries, avg, mg
Sheep pituitary—				
28B	50	—	9	51
”	—	100	3	15
”	50	6.25	3	100
”	50	12.50	3	113
”	50	25	6	177
”	50	50	9	187
”	50	100	3	195
F115	100	—	12	77
”	100	25	3	118
FSH333	1000	—	6	58
”	1000	25	3	84
Rat pituitary—				
RAP126	1	—	10	58
”	1	25	6	44
Sheep pituitary—		$Zn(OH)_2$		
28B	50	—	9	51
”	50	25	6	187
		$Mg(OH)_2$		
”	50	25	6	122
		$Fe(OH)_3$		
”	50	25	6	215

* Indicates mg equivalents of dry pituitary powder used in making the preparation.

recorded in Table II. These results show that 25 mg is almost as effective as 50 or 100 mg of aluminum hydroxide when given with 50 mg of a gonadotrophic preparation of this type.

The purified sheep pituitary gonadotrophic preparation (F115, Table II) when given in a dose of 100 mg produced ovaries that had an average weight of 77 mg and it was augmented but little by 25 mg of aluminum hydroxide as the ovaries obtained with this mixture had an average weight of 118 mg. Likewise, the follicle stimulating hormone preparation was not greatly augmented by aluminum hydroxide. The extract of dried rat pituitary glands was not augmented and the results suggest that there was inhibition.

The relatively crude sheep pituitary preparation 28B was effectively augmented by the hydroxides of magnesium, zinc, and iron; iron being somewhat more effective than magnesium or zinc.

The mechanism by which sheep pituitary gonadotrophin is made more effective by addition of insoluble hydroxides is not definitely known. However, a reasonable explanation can be based upon the well known fact that the hydroxides used in this work are known to adsorb most proteins from aqueous solution. In this connection we have evidence which shows that a suspension of aluminum hydroxide removes sheep pituitary gonadotrophin from solution. These facts make it seem probable that the mechanism consists of adsorption of the gonadotrophin from solution by the hydroxide and that on injecting the hydroxide-gonadotrophic complex into the rat, the gonadotrophin is released slowly but constantly from the hydroxide so that the ovary is stimulated continuously by the low concentration of the hormone in the blood. This explanation is the same as that given for the great augmenting effect produced by heme.[10,11] We believe that substances of this type simulate the way in which the pituitary gland constantly secretes small amounts of gonadotrophin into the blood.

The present methods of administering pituitary gonadotrophins result in ineffective utilization. That is, the maximum potential of an extract is not realized when injections are made at infrequent intervals because there is a relatively high concentration of the gonadotrophin in the blood for a short time, but during most of the time there is little or none present. This presents the problem of finding a method by which the maximum potential activity of gonadotrophic extracts can be made effective. As has been shown this can be done by increasing the number of injections from two to twenty-four times per day,[15] or by the use of heme,[10,11] but these methods are not practical for general purposes. The results obtained with the insoluble metallic hydroxides suggest that they might be utilized to make the hormones in pituitary preparations more effective without increasing the number of injections.

Summary: Aluminum, iron and zinc hydroxides were shown to be effective in increasing the effectiveness of sheep pituitary gonadotrophin while magnesium hydroxide was less effective. The effect of these hydroxides is explained on the basis of adsorption of the hormone by the hydroxide with slow release of the active substance from the site of injection.

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15044

Further Studies on Pituitary Responses to an Oxidative Inactivation Product of Estrone.

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It has been found that Westerfeld's[1] lactone, an oxidative inactivation product of crystalline estrone, causes adrenal and pituitary hypertrophy in mature male rats.[2] The adrenal response was shown to be mediated by the pituitary and transplantation of the hypophyses indicated increased release of gonadotropic as well as adrenotropic hormone. Control experiments with estrone gave evidence that these effects are not referable to the slight estrogenic activity of the lactone and that estrone elicits them only through the formation of some inactivation product, possibly the lactone or a related compound.

The present communication deals with investigation of the lactone in relation to 2 other pituitary responses to the estrogens which might conceivably be accountable to inactivation products rather than to estrogens *per se.* The first is the release of pituitary luteinizing hormone (L.H.) and the second the suppression, by prolonged administration, of pituitary follicle-stimulating hormone (F.S.H.).

Three different batches of the lactone were used. The first was an aliquot of Westerfeld's original preparation upon which he published the results establishing its chemical identity.[1] The other two were prepared by Dr. Alan Mather of Clark University, Worcester, Mass., following Dr. Westerfeld's general scheme of oxidation with H_2O_2 and purification. The biological properties of these 3 preparations were entirely similar. Dissolved in 0.005 N NaOH, their estrogenic potency approximated one-fifteenth that of estrone in the same medium, and was increased 3 to 5 fold by hydrolysis in the presence of the Zn and HCl.[2] In the open ring form (*i.e.,* in 0.005 N NaOH) they also showed the previously described pituitary-stimulating properties as well as the effect upon pituitary L.H. to be reported below. According to Dr. Mather's experience, one cannot be sure of acquiring this compound by this method. Three other preparations supplied by him, although derived from acetates whose melting points agreed quite closely with Westerfeld's, were completely non-estrogenic even after Zn-HCl hydrolysis, and failed to exhibit any pituitary-stimulating properties.

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[1] Westerfeld, W. W., *J. Biol. Chem.*, 1942, **143**, 177.

[2] Smith, O. W., *Endocrinol.*, 1944, **35**, 146.

TABLE I.
Enhancement of F.S.H. with the Lactone as Compared with Effect of Estrone in Corresponding Amounts of Estrogenic Activity.

		Effect on ovaries		
Injections to immature female rats	No. of rats	Avg wt, mg	Avg increase in wt over uninjected, littermate, %	No. of ovaries containing corpora lutea*
None	46	7.4		0
F.S.H. only	31	12.2	70	5 of 62 (8.1%)
F.S.H. plus the lactone, 10-20 γ, 1-3 R.U.	26	31.2	303	48 of 52 (92%)
F.S.H. plus estrone, 2-4 γ, 3-6 R.U.	27	12.6	68	4 of 54 (7.4%)

* Not all ovaries were sectioned. These figures refer to grossly viable discrete corpora lutea.

According to our own experience there is also considerable difficulty involved in eliminating contamination of the final product with unchanged estrone. Our studies of the biological properties of the lactone suggest that it may be of considerable therapeutic value and a more economical procedure for its preparation would be a real contribution. An even more practical aspect that should be investigated is the possibility that non-estrogenic compounds related to diethyl-stilbestrol may have these same biological properties.[3]

It has been shown that estrone stimulates the release of L.H. from the pituitaries of immature female rats, thereby augmenting the response to F.S.H. so that ovulation and luteinization take place.[4] In determining whether or not the lactone has this property, we experienced some difficulty in obtaining a source of F.S.H. with which Hisaw's experiments with estrone could be repeated. A partially purified powder prepared from alcohol precipitation of menopausal urine was found satisfactory and was used in most of the experiments to be reported. Only one of 3 extracts of pituitary glands was augmented by estrone, this being a Schering product prepared from sheep's pituitaries and non-luteinizing when given alone in twice the amount used for test. With both preparations the smallest amount which, when given in 6 injections over 3 days to 19- to 21-day-old, female rats, caused estrous smears on the fifth and sixth days, was considered 1 unit, and was used in testing for augmentation. Experiments were run on batches of 19- to 21-day-old littermates so that the ovaries of a rat that received no injections were compared with those of a littermate that received F.S.H. only and of another that received the same amount of F.S.H. plus the lactone. The F.S.H. was given in 6 injections over 3 days, the lactone in 6 injections on days 3, 4 and 5, and all animals autopsied on the seventh day, their ovaries weighed on a torsion balance and examined for corpora lutea. In order to be certain that enhancement with the lactone was not referable to its slight estrogenic activity, control experiments were run substituting estrone, in corresponding amounts of estrogenic potency, for the lactone. The results are summarized in Table I and demonstrate that 10 to 20 γ of the lactone augmented the ovarian response to the follicle-stimulating hormone and resulted in marked luteinization (Fig. 1), this effect being unassociated with the slight estrogenic activity of the oxidation product. Even when 10 to 20 γ of estrone were given (8 experiments, not included in Table I), the average increase in ovarian weight over that of control littermates was only 200% and only 50% of the ovaries contained corpora lutea. Since enhancement of F.S.H. with the lactone was not demonstrable in hypophysectomized animals* (6 experiments), we con-

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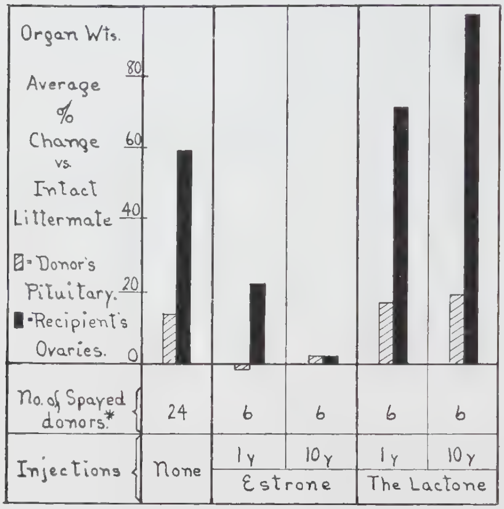
[3] Smith, O. W., and Smith, G. V., PROC. SOC. EXP. BIOL. AND MED., 1944, **57**, 198.

[4] Hisaw, F. L., Fevold, H. L., and Greep, R. O., *Proc. Assn. Res. Nerv. and Ment. Dis.*, 1936, **17**, 247.

* We are indebted to Dr. R. Tilsowitz, now of Schering Corporation, for hypophysectomizing for us the 28-day-old females used. In each experi-

TABLE II.
Effect of Estrone and the Lactone on Castration Changes of Pituitaries of Female Rats.



No. of Spayed donors.*	24	6	6	6	6
Injections	None	1 γ	10 γ	1 γ	10 γ
		Estrone		The Lactone	

* For each injected spayed donor there was one intact and one spayed control littermate.

clude that its effect is due to release of L.H. from the hypophysis. The fact that estrone is less active than the lactone in eliciting this response suggests that it exhibits this property indirectly, through the formation of the lactone or some similar inactivation product.

Castration results in hypertrophy of the pituitary and an increase in its transplantable gonadotropic activity associated with cytological changes indicating increased storage in the basophiles. These effects of castration are generally accepted as evidence of increased secretion of F.S.H. and may be prevented by prolonged administration of estrone. It seemed possible that inhibition of pituitary F.S.H., like the other pituitary effects of the estrogens, might be duplicated with the lactone on the supposition that this response also was accountable to breakdown products rather than to estrogens *per se*.

Twenty-four experiments, summarized in Table II, were run upon batches of 3 littermate, 25-day-old, female rats. In each experiment, 2 were spayed and the third left intact. One of the spayed animals served as uninjected control and the other received either estrone (a total dose of 1 or 10 γ) or the same amount of the lactone in 2 daily injections during the following 16 days. All were autopsied on the forty-second day of life, their pituitaries weighed on a torsion balance, ground in saline and transplanted in 6 injections over 3 days into littermate 19- to 21-day-old, female rats whose ovaries were weighed on the fifth day.

Castration caused a 2 to 17% increase in the weight of the pituitaries and a 42 to 93% increase in transplantable gonadotropic activity as measured by the weight of the ovaries of recipients. One gamma of estrone caused a partial and 10 γ a complete suppression of these castration changes, the results of pituitary transplant experiments being definitely significant, whereas the average weights of the pituitaries were indicative only. Representative experiments were run in which the pituitaries, instead of being transplanted, were sectioned and examined for us through the courtesy of Dr. R. O. Greep of the Harvard Dental School. Dr. Greep could find no cytological evidence for the influence of one gamma of estrone, but 10 γ completely prevented the histological changes following castration. In the experiments in which the lactone was given, on the other hand, there was certainly no suppression of castration changes and some evidence, from the results of pituitary transplant experiments, of an actual enhancement of gonadotropic secretion. The basophilia in the pituitaries of spayed rats that received 1 or 10 γ of the lactone was as marked, according to Dr. Greep, as in untreated spayed littermates. The suppression of F.S.H. by prolonged administration of estrone, therefore, may well be a direct effect. It is certainly not mediated by the formation of this particular oxidative inactivation product.

In this connection a single experiment may be cited for its confirmatory value. Two mature female rats having regular 4- to 5-day cycles were injected twice daily for 27 days, one receiving a total dose of 1 mg of estrone and the other a total dose of 1 mg of the lactone. The rat that received estrone was in

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ment F.S.H. alone was given to one and F.S.H. plus the lactone (10 γ) to the other of 2 littermates, injections being started the day after removal of the glands and the same procedure adhered to as in the experiments on intact animals.

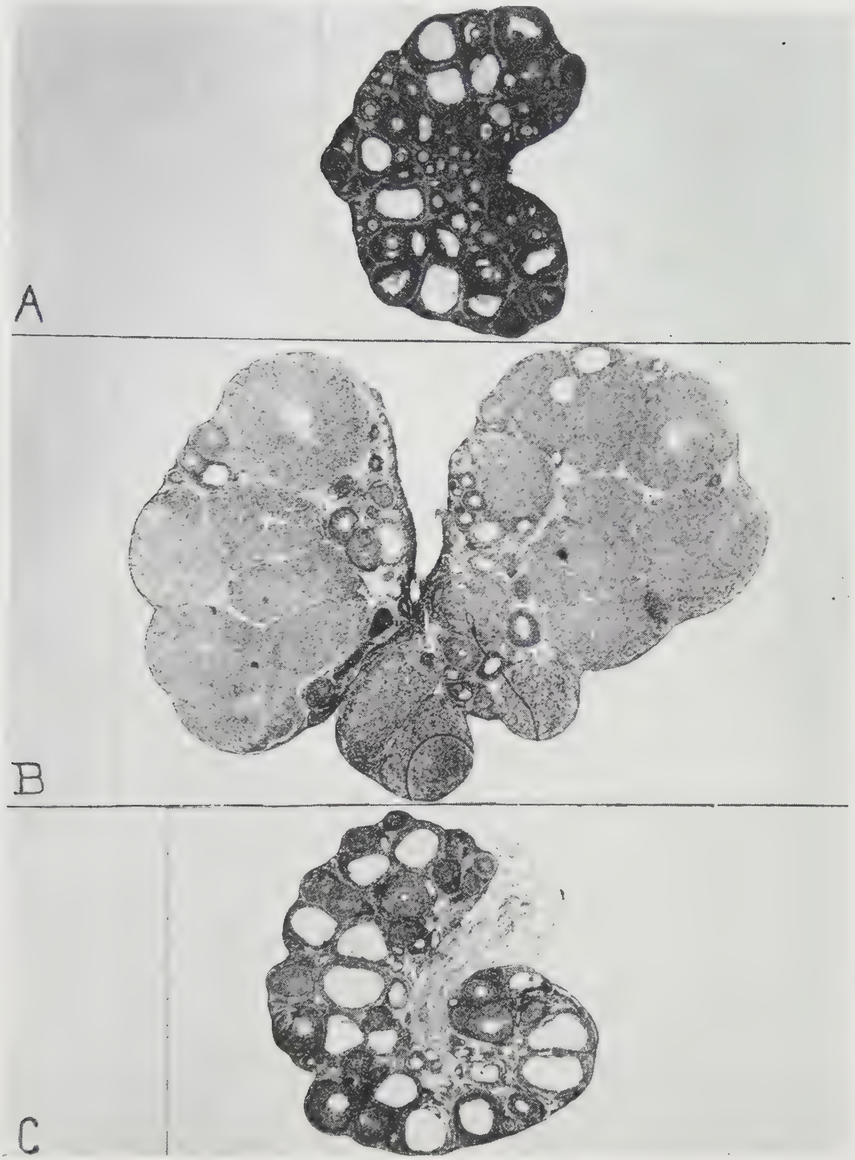

Fig. 1.

Luteinization of ovary of immature female rat with F.S.H. plus the lactone. One ovary from each of 3 littermates. A received F.S.H. (extract of menopausal urine) only. B received the same F.S.H. in the same amount plus 20 γ (2 R.U. of estrogenic potency) of the lactone. C received the same F.S.H. plus 2.0 γ (3 R.U.) of crystalline estrone.

constant estrus during injections. At autopsy on the 28th day its ovaries were atrophied (16.5 mg) and showed no evidence of secretory activity. The animal that received the lactone, on the other hand, continued to have 4- to 5-day cycles, although during the last 10 days all smears contained many nucleated and squamous cells. At autopsy on the twenty-eighth day the ovaries were normal in size (39.5 mg) and loaded with small, red corpora lutea.

Discussion. From the aspect of the therapeutic possibilities of the lactone, these and the previously reported observations[2] are of considerable interest. Hypophyseal extracts of animal origin have not proved very satisfactory as a means of establishing normal ovarian activity in women with functional disturbances. In certain situations estrogens have been apparently beneficial, presumably because of their pituitary-stimulating effect. As a pituitary stimulant, the lactone should be very much more useful than the estrogens, first, because it is practically non-estrogenic and would not be expected to cause such undesirable side effects as excess cervical secretion, breast changes and endometrial hyperplasia. Secondly, since, unlike estrone, it acts directly on the pituitary and is not influenced by the steroids (*viz.*, progesterone and testosterone[2]), which depress estrogen inactivation, the response to it would presumably be uninhibited by the patient's own secretion of steroid hormones. Finally, according to the above experiments, its prolonged administration should have no depressant effects upon pituitary or ovarian activity. As an inhibitor of F.S.H., on the other hand, it would presumably be ineffective.

Summary. Westerfeld's lactone, an oxidative inactivation product of crystalline estrone, stimulates the release of luteinizing hormone from the pituitaries of immature female rats, eliciting this response in smaller dosages than does estrone. Unlike estrone, it does not, upon prolonged administration, prevent the secretory or cytological changes in the pituitary which follow removal of the ovaries. Therapeutic implications are discussed.

15045 P

Effect of Fatty Acids on Acetylcholine Synthesis.*

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More acetylcholine is synthesized by the frog brain *in vitro* in the presence of serum than in its absence.[1,2] Performance of muscle work modifies the ability of serum to increase the acetylcholine synthesis.[3] Since muscle may utilize fats during performance of work[4-19] the

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* This investigation was aided by a grant from the John and Mary R. Markle Foundation.

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TABLE I.
Effect of the Substances on the Synthesis of Acetylcholine.

	Amt of acetylcholine synthesized in % of control*				
	Amts of the substances (mg) added to 100 mg frog brain				
Substance	3	0.3	0.03	0.003	0.0003
Glycerol	101	98	100	103	100
Saturated Fatty Acids:					
Formic acid	88	93	91	96	101
Acetic acid	102	101	104	102	102
Propionic acid	122	118	106	101	97
Butyric acid	131	119	110	102	102
Valeric acid	155	111	106	96	99
n-caproic acid	106	117	110	100	101
n-caprylic acid	64	96	97	103	101
Capric acid	53	80	104	98	102
Lauric acid	48	71	98	97	99
Palmitic acid	102	105	99	100	103
Stearic acid	91	102	97	103	100
Unsaturated Fatty Acids:					
Oleic acid	81	94	105	101	98
Linoleic acid	58	77	93	97	99
Aldehydes:					
Acetaldehyde	20	49	75	88	92
Glyceraldehyde	70	90	102	100	99
Ketones:					
Acetone	96	110	104	97	102
Acetoacetic acid	105	101	103	106	98
β-OH butyric acid	73	96	98	101	103

* Each value represents the average of 8 separate experiments. The S.E. of the mean for each value was less than ±5%. The amount of acetylcholine synthesized in μg per 100 mg frog brain, followed by the S.E. of the mean, was: 1.50 ± 0.044.

effect of fatty acids on the acetylcholine synthesis was investigated in the following.

Method. The effect of the substances on the acetylcholine synthesis was studied by the method described previously.[2] Mixtures containing varying amounts of the substances (pH corrected to 7.4), 100 mg minced fresh frog brain, 3 mg physostigmine salicylate, 4.8 mg glucose, and 3 cc Ringer's solution were shaken and incubated aerobically for 4 hours at 37°C. After incubation the amounts of acetylcholine synthesized were assayed biologically on the sensitized rectus abdominis muscle of the frog.

Results. The amounts of acetylcholine synthesized are given in Table I. Butyric-, propionic-, valeric-, and *n*-caproic acids increased the synthesis. These results suggest that during utilization of butyric- and propionic acids by the muscle these acids may exert a beneficial effect on the locally occurring acetylcholine synthesis.

Higher fatty acids decreased the synthesis of acetylcholine; palmitic-, and stearic acids seemed to have no effect on the synthesis, however, the lack of effect may have been due to the low solubility of these acids.

Endproducts of fat and carbohydrate metabolism (acetic acid, acetoacetate, acetone) did not modify the acetylcholine synthesis.

The synthesis was decreased in the presence of the unsaturated acids, aldehydes, and the ketone β-hydroxy butyric acid. This decrease of acetylcholine synthesis, if it occurs in the body, may contribute to the genesis of easy fatiguability observed during administration of large amounts of β-hydroxy butyric acid or during severe ketosis.

These results suggest that unsaturated and higher fatty acids contained in the blood may depress the synthesis of acetylcholine but the lower fatty acids occurring in the muscle either increase it or do not modify the synthesis.

Summary. 1. The effect of fatty acids and related substances on the synthesis of acetylcholine was investigated. 2. The synthesis was not modified in the presence of glycerol, acetic-, palmitic-, and stearic acid, acetone,

and acetoacetic acid. 3. The synthesis increased in the presence of butyric-, propionic-, valeric-, and *n*-caproic acid. 4. The synthesis decreased in the presence of formic-, *n*-caprylic-, capric-, lauric-, oleic-, linoleic acid, glyceraldehyde, acetylaldehyde, and β-hydroxy butyric acid.

15046

Modification of Serological Response to Infection with Murine Typhus by Previous Immunization with Epidemic Typhus Vaccine.

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It is well known that epidemic typhus cannot be differentiated from murine typhus by means of the non-specific Weil-Felix test since a high OX-19 agglutination titer may occur in both diseases. However, within the past few years, through improved technics and experimentation, both the specific complement fixation and agglutination tests have become of great value in differentiating these diseases. As was first shown in this laboratory, there is an antigenic fraction (soluble antigen) common to both types of rickettsiæ which reacts equally well in the complement fixation test with epidemic or murine antibodies.[1,2,3] However, by washing and centrifugation this antigen can be eliminated, leaving the purified rickettsial bodies. These latter constitute a highly specific antigen in the complement fixation test. Furthermore, these rickettsial suspensions have been found to be of great value as agglutinogen in the agglutination test. With technical improvements, recently described, this test has also been found to be of considerable value as a means of differentiating epidemic from murine infections.[4] As illustrations of the type of serological reactions found see Tables I and II which are representative cases selected from our series where the agent was isolated and identified and where serial serum specimens were available for examination.

It is noted that in epidemic typhus high complement-fixing titers are obtained with the purified epidemic rickettsial antigen and low or negative results with a purified murine rickettsial antigen. Conversely, in murine typhus high titers are obtained with a purified murine rickettsial antigen and low or negative results with a purified epidemic antigen. With regard to the rickettsial agglutination results higher titers are obtained with the homologous antigen than with the heterologous. However, it is to be noted that while there is cross agglutination the difference in titers is sufficient to permit clear differentiation.

During the past year we have received specimens from 147 cases of murine typhus occurring in soldiers stationed in the United States. Of these 135 gave a typical murine serological response comparable to that cited in Table II. However, 12 cases have given an unusual response. Table III records the serological results found in these cases.

In an attempt to determine the factors producing these atypical responses the 12 cases

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* Member of the U. S. A. Typhus Commission.

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TABLE I.
Serologic Results in a Non-vaccinated Individual Infected with Epidemic Typhus Fever.
Case 2724—Strain Isolated.

Day of disease	Weil-Felix*			Comp.-Fixation†		Rickettsial Aggl.	
	OX-19	OX-2	OX-K	Epidemic	Murine	Epidemic	Murine
8	1/20	0	0	1/40	0	1/80	0
11	1/320	1/40	0	0	0	1/160	1/40
13	1/2560	1/80	0	1/20	0	1/1280	1/320
15	1/2560	1/80	1/20	1/80	1/10	No serum	
19	1/2560	1/80	0	1/640	0	1/5120	1/1280
23	1/5120	1/80	0	1/2560	0	1/10,240	1/1280
25	1/2560	1/40	0	1/1280	0	1/5120	1/1280
27	1/1280	1/40	0	1/1280	0	1/5120	1/640
86	1/640	1/40	0	1/160	0	1/640	1/40
286	1/80	1/10	0	1/160	0	1/80	1/80

* Weil-Felix and rickettsial agglutination titers are recorded as final dilution.
† Complement fixation is recorded as initial dilution.

TABLE II
Serologic Results in a Non-vaccinated Individual Infected with Murine Typhus Fever.
Case I.—Strain Isolated.

Day of disease	Weil-Felix			Comp.-Fixation		Rickettsial Aggl.	
	OX-19	OX-2	OX-K	Epidemic	Murine	Epidemic	Murine
5	0	0	0	0	0	0	0
7	1/80	0	0	0	0	0	1/160
9	1/640	1/160	1/40	0	0	1/80	1/1280
11	1/640	1/160	1/80	0	0	1/160	1/2560
13	1/640	1/160	1/40	0	0	1/320	1/5120
14	1/640	1/80	1/40	0	0	1/320	1/5120
16	1/640	1/80	0	0	0	1/320	1/5120
18	1/320	0	0	0	1/80	1/320	1/5120
20	1/160	0	0	0	1/160	1/640	1/10,240
22	1/160	0	0	0	1/320	1/640	1/10,240
31	1/160	0	0	0	1/320	1/320	1/5120
75	0	0	0	1/40	1/320	1/40	1/1280
123	0	0	0	1/10	1/320	0	1/160

were investigated with some care. It was found that all of these patients had received typhus vaccine some time or other within the preceding 2 years. The vaccine used consisted of formalinized epidemic rickettsiæ with no murine rickettsiæ whatsoever. All cases occurred in regions of the United States where murine typhus alone is known to exist and in some instances other cases of murine typhus were present in the same camp at the time these cases were admitted to the hospital. The clinical diagnosis of murine typhus seems to have been well substantiated. All cases developed a typical rash. A rise in OX-19 titers occurred in the late febrile period and early convalescence in 6 cases, while most of the others showed high titers. The febrile period varied from 8 to 23 days with an average of about 14 days. Unfortunately, no attempt was made to isolate the agents.

From the figures given in Table III it is apparent that there is frequently a reversal between the results of the complement fixation tests and the agglutination tests and even the quantitative relationships are dissimilar to those previously found in individuals who had never received vaccine. It is observed that while many of these specimens showed higher epidemic complement fixation titers than was found with the murine antigen there was usually considerable cross fixation and a number of specimens showed equal titers to the 2 antigens. This is in contrast to what was found in non-vaccinated individuals infected with epidemic typhus where high epidemic titers are obtained and where con-

TABLE III.
Serologic Results Found in Individuals Who Were Previously Immunized with Epidemic Typhus Vaccine and Who Subsequently Contracted Murine Typhus.

Case	Day of disease	Typhus vac.	Compl.-Fix.		Rick. Agg.		Weil-Felix*		Date of onset	Where infected	Severity of infection
			Epi.	Mur.	Epi.	Mur.	OX-19	OX-2			
A	59	11/43	1280	320	80	640	80	80	1/1/44	Waycross, Ga.	Moderately severe
	90		1280	320	160	1280	0	0			
S	21	9/42	640	320	80	1280	2560	0	11/20/44	Ft. Benning, Ga.	" "
	42		320	320	80	640	640	0			
	58		160	160	80	320	640	0			
Si	27	5/27/44	A/C	A/C	2560	10240	160	40	7/19/44	Louisiana	Severe
	159		320	160	320	2560	160	40			
P	22	9/44	1280	640	320	640	160	0	12/17/44	Camp Gordon, Ga.	"
	50		1280	640	320	320	0	0			
B	18	11/44	160	40	5120	10240	640	0	11/23/44	Camp Shanks, N.Y.	Moderately severe
	21		160	160	5120	10240	320	0			
	26		160	160	5120	10240	0	0			
	31		160	160	5120	10240	0	0			
Be	18	8/44	640	160	5120	10240	1280	0	11/25/44	Birmingham, Ala.	" "
	70		320	160	160	1280	160	0			
Ben	38	10/44	80	80	1280	5120	640	40	10/24/44	Tampa, Fla.	Mild
K	30	9/44	160	80	5120	10240	80	0	9/18/44	Camp Miles Standish, Mass.	"
Ba	15	12/44	80	10	1280	5120	640	320	12/15/44	Robins Field, Ga.	"
F	44	1/44	80	80	640	5120	640	0	1/2/45	Austin, Texas	Moderately severe
	55		80	40	160	2560	1280	40			
T	45	7/44	1280	640	1280	5120	1280	160	1/14/45	San Antonio, Texas	Mild
Com	14	8/43	320	320	160	640	160	0	7/12/44	Hunter Field, Ga.	"
	23	Recall dose	A/C	A/C	1280	2560	160	40			
	24	5/44	320	320	1280	5120	80	40			
	30		320	160	320	2560	40	0			
	37		320	160	320	2560	0	0			

* Cases S, P, B, Ba, and T showed a rise in OX-19 titer when serum specimens were examined during the febrile period and early convalescence while the other cases showed high titers. These examinations were made in the hospitals where the patients were treated. It should be observed that the specimens which were available for this study were frequently obtained late in convalescence.

siderable cross fixation is not the rule. Similarly, the complement fixation results are not comparable with what is found in non-vaccinated individuals infected with murine typhus, for here again the homologous complement fixation titer is higher than the heterologous with little crossing (see Table II).

In contrast to the complement fixation results, the rickettsial agglutination usually showed a higher murine titer. However, as in the complement fixation results cross agglutination is marked and in many instances there is only a 2-fold difference in titer. The same type antibody response was observed in the cases reported irrespective of whether the epidemic typhus vaccine was administered two years or one week before the present attack of murine typhus. Insofar as no such reaction has been observed in vaccinated subjects who subsequently became infected with atypical pneumonia, measles, infectious hepatitis, smallpox, pneumonia, meningitis, or other virus, bacterial, protozoal infections, it is our belief that the unusual serological findings reported are to be attributed to the previous immunization with rickettsial products followed by an infection with murine rickettsiæ.

It is of interest to observe, as far as can be judged from the description of the symptoms and course of the disease, that the previous administration of the epidemic typhus vaccine had little or no effect upon the severity of the recent attack of murine typhus.

Summary. Twelve cases of murine typhus occurring in individuals who previously received epidemic typhus vaccine are reported. It appears that the previous administration of the epidemic typhus vaccine did not affect the course or severity of the murine disease. The serologic response was different from that found in non-vaccinated cases of epidemic or murine typhus. The Weil-Felix reaction does not seem to be affected, for a rise in titer or high titers were obtained in a majority of the cases. The difference occurred in the specific antibody response. While the epidemic complement fixation titers were higher than the murine titers, a number of specimens showed equal titers or considerable cross fixation. The murine agglutination titers were usually higher than the epidemic titer with considerable cross agglutination. This type of reaction has not been found in individuals who had received epidemic typhus vaccine and who subsequently became infected with bacterial, viral, or protozoal infections. Since many of our soldiers who have received epidemic typhus vaccine may subsequently develop murine typhus either abroad or after return to this country, it is obvious that we may expect to have a considerable number of future cases presenting the serological pattern described in this paper.

15047

Use of Vaginal Smears in Mating the Golden Hamster.

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Various procedures have been adopted in different laboratories for breeding the golden hamster, *Cricetus* (*Mesocricetus*) *auratus* Waterhouse. The general procedure involves placing the male with one or more females for varying lengths of time, then removing the male and watching for signs of pregnancy. The male may be housed with the female during 2 or 3 estrous cycles,[1] or for 7 or 8 days,[2] or the male may be placed in the cage with several females after 6:30 p.m. and, if mating activity is observed, the reacting animals are left together over night.[3] If no mating activity

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* Contribution No. 73.

[1] Peczenik, O., *J. Endocrin.*, 1942, **3**, 157.

[2] Sheehan, J. F., and Bruner, J. A., *Turtox News*, 1945, **23**, 65.

[3] Bond, C. R., *Physiol. Zool.*, 1945, **18**, 52.

TABLE I.

Pregnancy Record of Golden Hamsters Caged Together in Proestrus or Estrus and for 48 Hours Thereafter.

No. of the mating	Total matings	Pregnancies No.	Pregnancies % pregnancies	Matings per pregnancy
1st	72	39	54.2	1.85
2nd	18	8	44.4	2.25
3rd	6	2	33.3	3.00
4th	2	0	0.0	—
Total	98	49	50.0	2.00

occurs it is assumed that the female is not in heat and the male is removed immediately. These methods are designed primarily to minimize the time the male and female are caged together, thus keeping at a minimum injuries due to unsocial behavior. The methods are predicated on the probability that during the time of cohabitation the female will pass through at least one period of estrus during which fertilization might occur.

The purpose of the present paper is to report a simple and effective procedure for mating the golden hamster at the time the female is known to be approaching ovulation. The procedure depends on the inspection of vaginal smears from the females chosen for breeding.

When, at any time, it is desired to increase the size of our colony, vaginal smears are made from a sufficient number of the females in our breeding colony to produce the required number of litters. Samples of the vaginal contents are obtained with a sterile platinum loop previously moistened with physiological saline and introduced high in the vaginal lumen. The loop is then passed through a small drop of saline on a microscope slide and the smear is examined unstained. A male is then placed in the cage with each of those females revealed, by inspection of the smears, to be in proestrus or estrus. The animal is presumed to be in proestrus when the smear reveals a predominant number of large squamous cells the nuclei of which are not visible and when, in addition, typical high power fields (X440) exhibit not more than 2 leucocytes. A few oval epithelial cells with prominent nuclei may also be present. The animal is considered to be in estrus when field after field exhibits no leucocytes and when squamous cells alone are present. Illustrations of typical proestrous and estrous smears may be found in an earlier paper.[4] The male is withdrawn from the cage of the female 48 hours after being placed therein. No observation of the cohabiting couples is necessary. The date of introduction of the male is recorded and the cage containing the female is inspected at the time delivery is due. The earliest likely date of delivery will be the 14th day after the male is placed with the female. The latest likely delivery date will be 18 days after the male is placed with the female, *i. e.*, 16 days after the male is removed. Kupperman, Greenblatt, and Hair[5] have shown that in their colony 5% of the cases deliver on the 14th day, 85% on the 15th day, and 11% on the 16th day after the observation of spermatozoa.

Ninety-eight matings were made according to this procedure in all months of the year (Table I). "First mating" is herein defined as the first time that a male is introduced into the cage of a virgin female, or the first time a mating is attempted with a given female after a pregnancy. "Second mating" refers to the second attempt to obtain a pregnancy in a given female which has had no previous litter, or the second attempt to obtain a pregnancy after the previous delivery. From the table it will be noted that first matings resulted in pregnancies in 54.2% of the cases, while 50% of all matings resulted in pregnancies. We have found it uneconomical to maintain the female in the breeding colony after the third unsuccessful mating.

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[4] Kent, G. C., and Smith, R. A., *Anat. Rec.*, 1945, **92**, in press.

[5] Kupperman, H. S., Greenblatt, R. B., and Hair, L. Q., Abstract, *Anat. Rec.*, 1944, **88**, 441.

The procedure herein outlined has several advantages over other methods: 1. The number of injuries sustained by either member of a pair as a result of unsocial behavior is reduced to a minimum. 2. The procedure may be employed to produce litters at the approximate future date and in the approximate number required. 3. The date of delivery may be predicted within a few days. 4. The procedure may be carried out at any convenient hour of the night or day and it is unnecessary to watch for mating responses or to further observe the animals until the time of parturition draws near.

Peczenik[1] states that the female may be recognized as being in heat when a thick mucus may be squeezed from the vagina. This is not a reliable criterion for determining the proper time for mating among our animals. The animals are still receptive when this thick mucus smear first occurs high in the vagina. At this time the animal is in metestrus, a phase characterized by the presence almost exclusively of elongated squamous cells in abundance and occupying from 4 to 8 hours only. This particular smear picture has not been widely observed. Peczenik states that at this time the animal has already ovulated. It may be that these few hours are the precise time when matings prove fertile. Kupperman, Greenblatt, and Hair[5] state that in their colony mating occurs without exception at this particular time ("in the transitional period between the cornified cell stage and the thick, dense smear of nucleated and cornified cells"). Nevertheless thick mucus may be squeezed from the vagina during early diestrus as well, when matings will not prove fertile. To test the criterion of thick mucus discharge as indicative of the proper time for mating we attempted 18 matings at the time of the cycle when the thick mucus is first evident. The males were left with the females for 48 hours and then withdrawn. We obtained one litter only by this method. In the latter case the vaginal smear of the female indicated estrus on the date the male was removed.

Enders[6] has successfully employed the microscope as an adjunct to breeding mink. We have found the method herein described most efficacious in maintaining the size of our hamster colony, and providing litters on the approximate date when they are required while at the same time eliminating animal injury which often results from unsocial behavior.

Summary. In the experiments herein reported vaginal smears have been utilized in determining the proper time for mating golden hamsters. A male hamster is placed in the cage with each female hamster which is found to be in either proestrus or estrus upon inspection of its vaginal smear. The male is caged with the female for 48 hours and is then removed. Injury due to unsocial behavior of the animals is minimized by this procedure. Forty-nine pregnancies were secured from a total of 98 matings in the present experiments. One pregnancy in 2 matings is considered to be a satisfactory conception rate for this animal under laboratory conditions.

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[6] Enders, R. K., *Am. Fur Breeder*, 1939, **11**, 6.

15048 P

The Need for Manganese in Bone Development by the Rat.*

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Shils and McCollum,[1] by feeding a high calcium-phosphorus diet low in manganese, obtained results indicating that the surviving young of deficient females developed skeletal abnormalities. Wachtel, Elvehjem, and Hart[2] reported a reduction in the ash content, density, and length of bones of manganese-deficient rats, fed a high calcium-phosphorus diet. Whether these results were caused by the retarding effect of manganese deficiency on growth in general or by a specific effect upon bone formation could not be determined from the evidence. Studies were undertaken, therefore, to determine if, by feeding a similar diet, evidence could be obtained of abnormal bone formation in rats in which the gains in weight of pairmates were equalized throughout the experimental period.

Rats of the Sprague-Dawley strain were used in these studies. All mothers were placed on the low-manganese diet 2 weeks after the birth of the litters to prevent excessive storage of manganese by the young prior to the experimental period. At 4 weeks of age the rats were paired according to weight and placed on the experimental diets. Each rat was weighed daily and the feed consumption of the rat on the high-manganese diet was limited so that it gained in weight at the same rate as its pairmate. The rats on the low-manganese diet were fed *ad libitum.*

The basal diet was a modification of low-manganese diet 21 devised by Shils and McCollum.[1] Additional $Ca_3(PO_4)_2$ was added so that the diet contained approximately 2% calcium and 1% phosphorus. The high-manganese diet contained a supplement of 0.06% manganese as $MnCl_2 \cdot 4H_2O$.

The rats were killed at 76-87 days of age and the left and right femur, tibia, and humerus dissected out and cleaned. Length, density, breaking strength, and bone phosphatase[3] were determinted on the fresh bone. The tibiae were used for the determination of bone ash. The ash samples were dissolved in 1:4 HCl and aliquots of these solutions were used for analyses of calcium,[4] phosphorus,[5] and magnesium.[6]

The results obtained are presented in Table I. The bones of the rats on the high-manganese diet were slightly longer than those on the low-manganese diet. When the lengths of the tibiae of pairmates of the same weight were compared and analyzed statistically by Student's method for paired data, the odds that the difference was significant were 185:1.

The density of the fresh bones of manganese-deficient rats was somewhat lower than that of their pairmates on the high-manganese diet. The odds that the difference was real were 70:1, showing that the difference, though slight, was significant. The

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[1] Shils, M. E., and McCollum, E. V., *J. Nutrition,* 1943, **26**, 1.

[2] Wachtel, L. W., Elvehjem, C. A., and Hart, E. B., *Am. J. Physiol.,* 1943, **140**, 72.

[3] Wiese, A. C., Johnson, B. C., Elvehjem, C. A., Hart, E. B., and Halpin, J. G., *J. Biol. Chem.,* 1939, **127**, 411.

[4] Pierce, W. C., and Haenisch, E. L., *Quantitative Analysis,* 1940, 367, John Wiley and Sons, Inc., New York.

[5] Allen, R. J. L., *Biochem. J.,* 1940, **34**, 858.

[6] Weeks, M. E., and Todd, J. R., *Ind. and Eng. Chem.* (Anal. Ed.), 1943, **15**, 297.

TABLE I.
Summary of Data.

	Females		Males	
Manganese content of diet	Low	High	Low	High
No. of rats	13	13	5	5
Final weight, g	190.8	191.1	237.0	237.6
Length of fresh tibia, cm*	3.38	3.43	3.44	3.49
Density of fresh bone, g/cc†	1.536	1.570	1.461	1.479
Breaking strength of femur, kg	7.90	9.35	7.89	9.91
Phosphatase, units/g fresh bone	12.69	14.46	17.02	17.43

* Avg of 2 bones.
† Avg of all bones.

lower density of the bones of the manganese-deficient rats observed in this study cannot be explained by a reduction in ash content of the bones since the percentage ash was the same for both groups. Caskey, Norris, and Heuser (unpublished results) found that the bones of manganese-deficient chicks contained slightly more fat and less water than those of normal chicks. If the same is true of the rat, it could explain the lower density without corresponding decrease in ash content.

The percentage of calcium and phosphorus in the fat-free dry bone was not lowered in the manganese-deficient animals. These results agree with those of Wachtel *et al.*[2] The percentage of magnesium was also the same for both groups.

The breaking strength of the bones of manganese-deficient rats was much less than those of the controls. The odds that the difference was significant were greater than 999:1. These results are in agreement with those reported by Smith, Medlicott, and Ellis[7] for the rabbit. The difference may be due in part to the lower density of the bones of deficient rats or to structural changes in the matrix of the bone, not affecting density and mineral content. The possibility also exists that there may be changes in the crystal structure of the bone salts.

The amount of phosphatase activity of the fresh bone was less in the manganese-deficient rats. The difference was found to be highly significant with odds of 830:1. These results are not in agreement with those of Wachtel *et al.*[2]

Summary. The length, density, breaking strength, and phosphatase content of the bones of manganese-deficient rats were lower than in pairmates of the same weight receiving adequate manganese. There was no difference between the two groups in percentage of ash, calcium, phosphorus, and magnesium in the fat-free dry bone.

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[7] Smith, S. E., Medlicott, M., and Ellis, G. F., *Arch. Biochem.*, 1944, **4**, 281.

15049

A Method for Determination of Streptomycin in Body Fluids.

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The introduction of streptomycin as a chemotherapeutic agent for the treatment of bacterial infections[1-4] has emphasized the need for a quantitative method for the determination of the drug in body fluids.

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[1] Jones, D., Metzger, H. J., Schatz, A., and Waksman, S. A., *Science*, 1944, **100**, 103.

[2] Robinson, H. J., Smith, D. G., and Graessle, O. E., PROC. SOC. EXP. BIOL. AND MED., 1944, **57**, 266.

In a recent communication, Foster and Woodruff described a method for the determination of streptothricin in aqueous solution.[5] Attempts to apply this procedure directly to the quantitative determination of streptomycin in blood, urine and body tissues were not satisfactory for, among other things, it was found that the blood of certain animal species, as well as man, contains a substance which produced a marked inhibitory effect upon the test organism *(Bacillus subtilis)*. Frequently this substance was present in amounts sufficient to produce a zone of inhibition larger than that obtained with low concentrations of streptomycin. Furthermore, the method of Foster and Woodruff, when applied to the assay of streptomycin, was not sufficiently sensitive to measure accurately concentrations of the drug in serum below 20 units per cc.

The foregoing difficulties were largely eliminated by the use of a streptomycin sensitive organism *(Staphylococcus aureus SM)* which was not inhibited by normal blood, by increasing the pH of the medium and by decreasing the salt concentration. These two latter factors were shown by Foster and Woodruff to influence the activity of streptothricin and later[6] were shown to enhance the activity of streptomycin in culture fluids, etc., when *Bacillus subtilis* was employed as the test organism.

The assay described in this communication is suitable for the determination of streptomycin in blood, urine and tissue fluids of mice, rats, rabbits, dogs, monkeys and man.

Materials. A strain of *Staphylococcus aureus SM* is used as the test organism. The culture is maintained in a uniform condition by daily transfer in F.D.A. nutrient broth at 37°C. The assay medium is a modified F.D.A. nutrient agar.* Including the beveled glass cylinders known as penicylinders,† the glassware required is ordinary standard bacteriological equipment.

Procedure. Using a 6-hour culture of the test organism grown in F.D.A. broth, a serial 10-fold dilution is made through 10^{-4} in broth. A final dilution to 10^{-5} is then made directly into the melted nutrient agar which has previously been cooled to 45°C. With the aid of a calibrated wide mouth pipette, 10 cc of this melted seeded agar is added to each of a series of petri dishes and these are set aside until the agar solidifies. Next, the beveled glass cylinders are warmed slightly by passage through a Bunsen flame and immediately placed upon the surface of the agar plate. In this manner a seal between the glass cylinder and the agar is effected. Four such cylinders are placed in each agar plate. The glass cylinders are then filled with the properly diluted samples for assay and the plates incubated at 30°C for 16-18 hours. At the end of this period the diameters of the zones of inhibition are measured in millimeters.

Assay of Blood, Urine and Tissue Extracts. In determining the concentration of streptomycin in blood, it is necessary to construct a standard curve of reference for each assay using an aliquot of the drug solution which is being administered to the patient. This is done by making dilutions of streptomycin containing 1, 2, 4, 8, and 16 units per cc in serum obtained from each individual prior to treatment or from any normal donor. The diameters of the resulting zones of inhibition are plotted as ordinates (arithmetic scale) against the concentrations of streptomycin as abscissa (logarithmic scale). The values obtained in a typical assay are shown in Table I, and the standard curve obtained therefrom in Fig. 1.

Test serums containing unknown amounts

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[3] Feldman, W. H., and Hinshaw, H. C., *Proc. Staff Meet., Mayo Clinic*, 1944, **19**, 596.

[4] Heilman, F. R., *Proc. Staff Meet., Mayo Clinic*, 1944, **19**, 553.

[5] Foster, J. W., and Woodruff, H. B., *J. Bact.*, 1943, **45**, 408.

[6] Foster, J. W., and Hendlin, D., to be published.

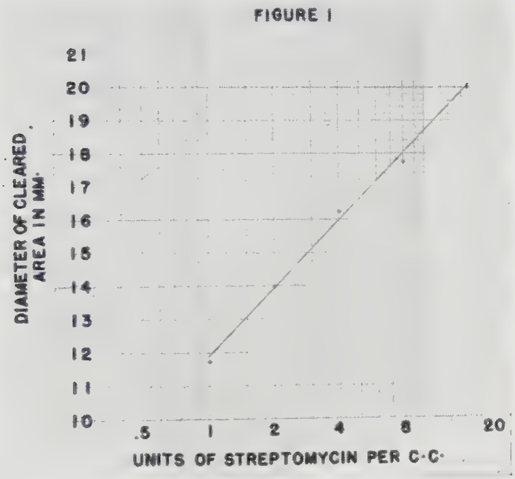
* Peptone (siccum-Armour)	10.0 g
Bacto Beef Extract (Difco)	5.0 ”
Salt (NaCl)	2.5 ”
Agar-agar	10.0 ”
Distilled water	1,000 cc

Adjusted to pH 7.5-8.0 with N/1 NaOH

† This is a trade name for a special type of glass cylinder developed for the assay of penicillin.

TABLE I.
Diameters of Cleared Areas Produced by a Streptomycin Standard with Human Serum as the Diluent.

Units per cc	1 mm	2 mm	4 mm	8 mm	16 mm
Plate 1	12.0	14.0	16.5	18.0	20.0
Plate 2	11.5	14.0	16.0	17.5	20.0
Avg	11.75	14.0	16.25	17.75	20.0



A standard curve for streptomycin in human serum.

of the drug are diluted with any normal human serum to contain approximately 5 to 15 units per cc. This will insure a reading which will fall on the standard curve. Dilutions of the test sample as well as each unitage level of the standard should be run in duplicate. The drug concentration in the serum sample is obtained by determining from the standard curve the streptomycin concentration corresponding to the diameter of the zone of inhibition and correcting for the dilution.

In the assay procedure for urine, feces or tissue extracts distilled water replaces serum as the diluent.

The results of a study of the distribution of streptomycin in blood, urine and tissue fluids in a variety of animal species will be reported elsewhere.

Statistical Analysis of the Method.‡ The curve relating unitage of streptomycin per cc in the cylinder to the diameter of the cleared area on the plate was investigated in three assays with several individual human sera, water, and certain combinations of serum and water as diluents for the drug. The data in these assays were analyzed in accord with methods described by Bliss for the biological assay of drug potency.[7,8]

These assays showed that the diameter of the cleared area in millimeters could be plotted as a straight line against the logarithm of the unitage in the range from 1 or 2 units to 20 units per cc. There was no significant change in sensitivity of the assay with change in unitage level. Individual or pooled sera could be used interchangeably as diluent without affecting relative unitage of streptomycin within a given assay.

The apparent unitage of streptomycin when assayed in a 2-fold dilution of serum with water was 89 ± 6%§ of the unitage in serum alone, and the apparent unitage in the 4-fold dilution of serum with water was 82 ± 6% of the unitage in serum. The apparent unitage in water, however, was 79 ± 8% and 57 ± 7% of the unitage with serum as the diluent in 2 different assays.

The slopes of the standard curves in the above 3 assays were 8.6, 6.7 and 5.9 (mm change in zonation per 10-fold change in unitage level). All the curves within each assay were parallel regardless of diluent, although slight deviation from linearity was observed at the 1 unit level in the first assay and some curvature was present in the third assay. The inherent precision of the method, as indicated by $s/b = 0.068$, 0.052 and 0.055 for the 3 assays, is quite satisfactory for a biological assay.[9] The variation between plates was of borderline significance.

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‡ The authors wish to thank Dr. Walther H. Ott for permission to include in this paper his report on the statistical analysis of the data.

[7] Bliss, C. I., and Marks, H. P., *Quart. J. Pharm. and Pharmacol.*, 1939, **12**, 182.

[8] Bliss, C. I., *J. Am. Stat. Assn.*, 1944, **39**, 479.

§ Standard error.

[9] Bliss, C. I., and Cattell, M., *Ann. Rev. Physiol.*, 1943, **5**, 479.

15050

Relation of Glycemia Level to Anoxic Survival of Primitive Respiratory Center in the Young Rat.

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Recently Selle[1] has shown that glucose and insulin have practically the reverse effects on the survival of the isolated respiratory center in the decapitated young rat head. It was likewise demonstrated[2] that glycotropic substances such as adrenaline and pituitary extract prolong, while others such as ephedrine, insulin, and iodoacetic acid, shorten the survival time of the respiratory center of the isolated young rat head. Starvation shortens survival time, presumably as less glucose remains available. It has recently been reported[3] that the aromatic sympathomimetic amines causing hyperglycemia without central excitation markedly prolong survival of the respiratory center while those causing central excitation concurrent with hyperglycemia shorten total survival time even though more gasps occur. This latter can be explained by the fact that the excited center discharges impulses more rapidly (lowered CO_2 threshold) resulting in quicker depletion of the available energy substratum.

In order to correlate survival time with glycemic level in the absence of neurogenic effects 113 young rats were decapitated by methods previously described[2] and the length of survival time determined by the measured duration of gasping. Blood sugar of each rat at the time of decapitation was determined by the Folin-Wu micro method[4] using a 0.1 ml sample of blood. Different blood sugar levels were achieved by (a) starvation, (b) glucose feeding (intragastrically), and (c) in 9 instances by injection of insulin intraperitoneally in order to reach extremely low blood sugar levels. It should be mentioned that with starvation plus insulin we were able to lower blood sugars to 19 mg % without convulsions in the young rats (approximately 24 days of age). All blood glucose readings were made objectively with a photelometer which had been calibrated with carefully prepared fresh dextrose standards. Intragastric feeding was done by a spinal puncture needle the end of which had been rounded and buffed to smoothness, with very few cases of failure in passing it down the oesophagus. All failures were discarded. By using relatively large doses of glucose (2 ml of 25%) we were able to reach blood sugar levels as high as 485 mg % but the results showed that prolongation of survival by glucose feeding was insignificant above the 180 mg level; in other words even though the glucose level may be high the rat apparently is unable to utilize a surplus above 180 mg %. This undoubtedly is due to the fact that the ischemic respiratory center is a dying center which is unable to utilize the extra fuel substance. Perhaps the very young (newborn) animal could utilize this extra energy to better advantage than the older one.[5,6]

Considerable individual differences in survival time appear and at first led us to believe that no correlation existed between glycemic level and survival time. However, if all the cases are plotted graphically, the "scatter graph" so produced shows a definite trend in one direction. Fig. 1 illustrates this relationship in 113 animals of a limited weight range. As far as possible we used rats weighing from

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[2] Hiestand, W. A., Tschirgi, R. D., and Miller, H. R., *Am. J. Physiol.*, 1944, **142**, 153.

[3] Tschirgi, R. D., and Hiestand, W. A., *Anat. Rec.*, 1945, **89**, 15.

[4] Folin, O., and Wu, H., *J. Biol. Chem.*, 1928, **77**, 2.

[5] Himwich, H. E., Bernstein, A. O., Herrlich, H., Chesler, A., and Fazekas, J. F., *Am. J. Physiol.*, 1942, **135**, 387.

[6] Himwich, H. E., Fazekas, J. F., and Homburger, E., *Endocrinol.*, 1943, **33**, 96.

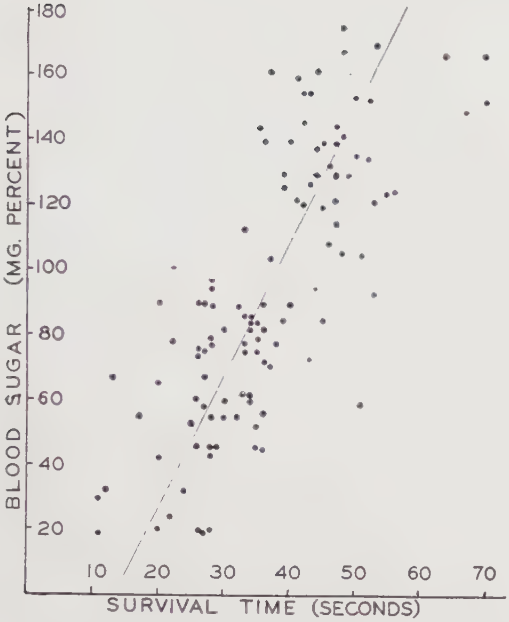

FIG. 1.
Showing trend in relationship between blood sugar levels below 180 mg% and respiratory center survival in 113 isolated young rat heads. The correlation for these data (Pearson r) is .737 ± .03.

TABLE I.
Weights, Survival Time, and Blood Sugar Levels of 10 Young Rats Fed Glucose Intragastrically. These are not included in Fig. 1.

Wt of rat, g	Survival time (sec)	Blood sugar (mg%)
48	55	485
45	50	458
47	67	438
48	61	403
45	59	395
55	36	322
42	55	290
43	59	282
55	44	215
55	52	205

50 to 60 g and of nearly the same age, the animals thus being young enough to have the second (anaerobic) series of gasps. As some were starved to reduce their blood sugar their weight fell, in some cases as much as 30%. Some given food *ad libitum* temporarily increased in weight. All weights listed are those taken immediately before decapitation.

As has been previously shown [2,5,6,7] the second series of gasps of the isolated head is anaerobic whereas the first series is oxidative. Therefore the duration of survival of the respiratory center is limited by its ability to continue using the anaerobic (glycolytic) energy. It is therefore reasonable that glycemic level should be a major factor in survival. It has been shown[6] that the survival period of the anoxic infant (rat or cat) but not that of the adult is prolonged by hyperglycemia. This is due to the inability of the adult to sustain a higher rate of cerebral metabolism from glycolysis and no doubt accounts for the absence of the second or anaerobic series of gasps in (most) adult rats. It appears from this and work of others that survival of the center depends upon 3 factors: (a) amount or availability of glucose, (b) state of activity of the center, and (c) the age of the animal. The last factor no doubt involves the animal's ability to utilize glucose as an anaerobic substratum which has been well explained by Himwich *et al.*[6]

Summary. The survival time (period of gasping) of the respiratory center and blood sugar level of 123 young rats were determined. A rough correlation exists between the two and it is concluded that glycemia is a significant factor in survival of the primitive respiratory center. Hyperglycemia above 200 mg % does not significantly prolong survival time beyond that of the 180 mg level.

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[7] Selle, W. A., PROC. SOC. EXP. BIOL. AND MED., 1943, **54**, 291.

15051

Nutritive Value of Dextri-Maltose Determined by the Single-Food Choice Method.

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The single-food choice method has been used in previous studies[1,2,3] to determine the nutritive values of dextrose, sucrose, casein, and a variety of other substances, together with the effect produced on their utilization by thiamine hydrochloride. Rats of a standard age were kept on a complete stock diet until they had reached a standard weight and then were placed on a diet which consisted of only one food-stuff. The survival times and daily running activity of the rats on the single food were taken as criteria of the nutritive value of each food-stuff. On dextrose alone the rats lived on the average 37 days and with access to 0.02% solution of thiamine hydrochloride 74 days; on sucrose alone they lived on the average 37 days and with access to thiamine solution 56 days; on casein alone they lived on the average 33 days and with access to the thiamine solution 55 days.

This method has now been applied to the study of the nutritive value of dextri-maltose.

Methods. Female rats were kept in individual activity cages which consisted of a revolving drum with a cyclometer and a living compartment with a nonspillable food cup and a graduated inverted water bottle. The rats were placed in the cages at 40 to 50 days of age, weighing at the time 80 to 100 g. They received our stock diet* for a minimum of 15 to 20 days, until they had reached weights between 120 and 159 g. Then the stock diet was replaced in the food cup by dextri-maltose.†

Daily observations were made of running activity, food and water intake, and of the vaginal smears; and body weight was recorded weekly.

Twelve tame domestic female Norway rats were used in these experiments.

Results. Survival Time—Table I shows the individual and average survival times of the rats kept on the single food (A) without access to thiamine hydrochloride and (B) with access to thiamine hydrochloride. For purposes of comparison with dextri-maltose, records (from previous work) have been included for dextrose, maltose, dextrin, corn starch, sucrose, and casein. On the single food without thiamine the survival times for the corn starch and sugars ranged from 31 days for corn starch to 37 days for dextrose. The survival times for casein averaged 33 days and for sucrose 37 days.

In marked contrast the survival times for the rats on dextri-maltose averaged 85 days, over twice as long as for starch or any of the individual sugars.

When given access to thiamine hydrochloride the rats on corn starch survived on the average 86 days, almost exactly as long as the rats on dextri-maltose. The rats on dextrose survived a shorter time, 74 days, and on casein and sucrose a still shorter time, 55 and 56 days respectively.

Food Intake—Since we know most about the effects of a single-food dextrose diet, we have made all of the comparisons of dextri-maltose with this sugar. Fig. 1A shows the average daily food intake for the 12 rats on dextri-maltose compared to values for 21 rats on dextrose, and 12 rats on dextrose with access to the 0.02% solution of thiamine hydrochloride. The intake of dextri-maltose was

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* The stock diet used contained graham flour 72.5%, skim milk powder (Breadlac) 10.0%, casein (No. 30, Labco) 10.0%, butter 5.0%, calcium carbonate 1.5%, and sodium chloride 1.0%.

[1] Richter, C. P., and Rice, K. K., *Am. J. Physiol.*, 1942, **137**, 573.

[2] Richter, C. P., and Rice, K. K., *Am. J. Physiol.*, 1944, **141**, 346.

[3] Richter, C. P., and Rice, K. K., *Am. J. Physiol.*, 1945, **143**, 336.

† Dextri-maltose No. 2 furnished by Mead Johnson, Evansville, Indiana.

TABLE I.

Diet	No. of rats	Sex of rats	Avg age at start of single food (days)	Avg wt at start of single food, g	Individual survival times (days)	Avg survival times (days)
A. Single Food Without Access to Thiamine Hydrochloride.						
Control rats (no food)	12	♀	60 (58-68)	133 (119-149)	3,4,4,4,4,4,4,4,4,4,5,5	4
Corn Starch	8	♀	63 (61-70)	134 (126-145)	25,29,29,30,31,34,35,35	31
Casein	16	♀	63 (55-69)	132 (124-145)	26,26,26,27,28,28,32,32, 32,33,33,34,39,45,46	33
Maltose	8	♀	67 (62-71)	142 (137-152)	31,31,32,35,35,36,37,39	34
Dextrin	5	♀	71 (67-73)	140 (129-150)	31,31,37,39,43	36
Dextrose	21	♀	64 (56-71)	137 (120-149)	28,29,32,33,33,34,35,36, 36,36,37,37,38,39,39,40, 40,41,42,42,54	37
Sucrose	11	♀	63 (56-66)	133 (124-145)	29,31,33,34,34,38,39,40, 42,46	37
Dextri-Maltose	12	♀	67 (64-73)	141 (120-159)	57,69,73,81,83,86,90,91, 93,96,98,99	85
B. Single Food with Access to Thiamine Hydrochloride.						
Casein	14	♀	60 (53-63)	137 (119-149)	36,40,41,42,42,52,55,56, 60,64,65,68,70,72	55
Sucrose	13	♀	62 (55-66)	138 (127-148)	35,40,45,47,47,50,55,57, 66,69,70,75,77	56
Dextrose	12	♀	62 (58-66)	139 (130-148)	62,65,67,72,73,74,74,75, 76,76,87,87	74
Corn Starch	5	♀	58 (55-69)	132 (122-141)	81,82,83,92,94	86

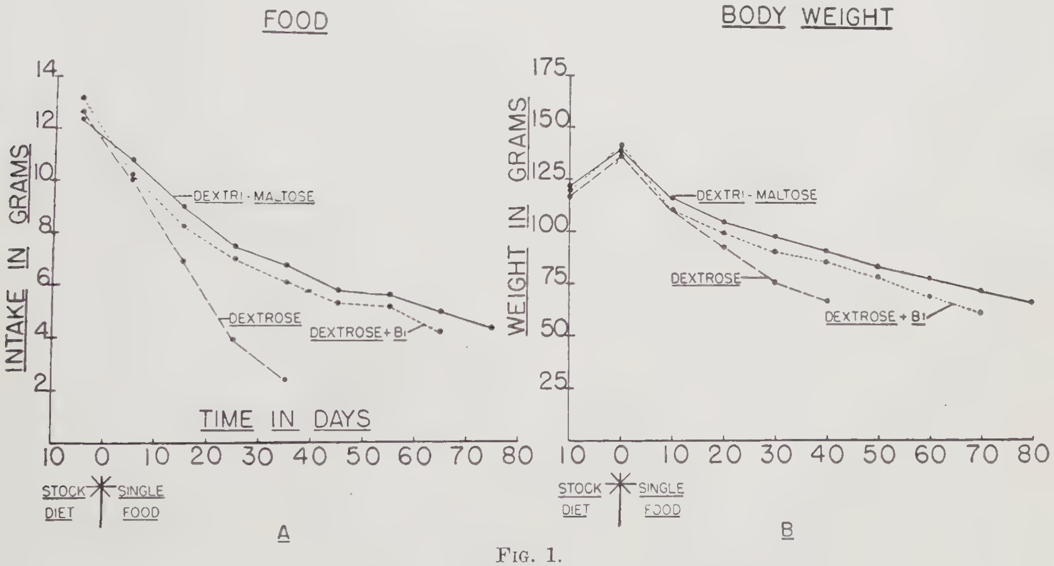

FIG. 1.

definitely higher than the intake of dextrose, either with or without thiamine.

Body Weight—Fig. 1B shows the average body weight of the 3 groups of rats whose respective food intake curves were shown in Fig. 1A. The dextri-maltose rats lost weight at a considerably slower rate than did the rats on dextrose and thiamine.

Activity—The average daily running activity of the rats on dextri-maltose closely approximated that of the rats on dextrose with access to thiamine. The rats remained quite active, running as much as 15 miles per day 60 days after the start of the dextri-maltose diet.

Water Intake—The water intake curves of the dextri-maltose and dextrose plus thiamine groups of rats were essentially the same.

Vaginal Smears—The effects produced on the vaginal smears also were much the same as those found in the dextrose rats which had access to thiamine. The 4-5 day oestrous cycles disappeared after 6 to 8 days leaving only dioestrous smears.

Discussion—The results of these experiments showed that dextri-maltose must contain some substance or substances that are not present either in dextrose or maltose, or even in the unhydrolyzed corn starch. The evidence at hand indicates that thiamine must be one of these substances.‡

In the first place thiamine is the only substance known at the present time which will prolong the survival time of rats kept on a single-food carbohydrate diet. Other members of the vitamin B complex do not have this effect, nor does salt.[4] Further, the average survival time of 85 days of the rats on dextri-maltose without added thiamine closely approximates the survival times of the rats on the other corn sugars and starches when given access to thiamine. In general thiamine slightly more than doubles the survival time of the rats kept on the corn sugars and starches.

From single-food choice experiments we can obtain only an indirect estimate of the amount of thiamine which is probably present in the dextri-maltose. Rats kept on dextrose took approximately 20 γ (allowing 1 cc for evaporation of solution) of thiamine per gram of food. If we assume that approximately the same amount of thiamine is needed for the utilization of dextri-maltose as for dextrose, then each gram of dextri-maltose might contain as much as 20 γ per gram.

With the thiochrome method, Holt[5] and Emerson and Obermeyer[6] found that dextri-maltose contains only 0.7 and 0.9 γ per gram respectively. The values estimated by the single-food choice method undoubtedly are far too high but it is possible also that the values determined by the thiochrome method may be too low.§

It appears likely, however, that dextri-maltose contains at least the minimum amount of thiamine necessary for its full utilization. In other words, more thiamine would not have helped the animal to utilize more dextri-maltose, nor would it have lengthened the survival times. The evidence for this statement is found in the observation that fortification of dextrose with 25 mg of thiamine per 100 g of food, that is, about 380 times as much as the rats on dextrose alone took voluntarily, did not increase the survival time. Six rats which received the thiamine mixed with the dextrose lived on the average 76 days.

The slightly longer average survival times of the rats on dextri-maltose as compared to that of the rats on dextrose with access to thiamine, also their slightly higher food intake and body weight, may indicate that dextri-maltose contains nutritive substances in addition to the thiamine. Emerson and Obermeyer[6] on the basis of results obtained with an entirely different type of experiment also arrived at this conclusion.

Najjar and Holt[7,8] used dextri-maltose as the source of carbohydrate in a thiamine deficient diet‖ which they gave to 9 young adolescent males over an 18-21 month period. The present results may explain in part the long period required to bring out the thiamine deficiency symptoms in most of the subjects, and that one failed even after 20 months to develop deficiency symptoms.

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‡ After the completion of the above experiments 6 rats were kept on a single-food choice diet of dextri-maltose in which all, or nearly all, of the thiamine had been destroyed. These rats survived 31, 35, 35, 35, 52, 54 days respectively, or 40 days on the average. This survival time closely approximates that of the rats kept on dextrose without access to thiamine.

[4] Holt, L. E., Jr., and Kajdi, C. N., *Bull. J. H. H.*, 1944, **74**, 142.

[5] Holt, L. E., Jr., *Fed. Proc.*, 1944, **3**, 171.

[6] Emerson, G. A., and Obermeyer, H. G., PROC. SOC. EXP. BIOL. AND MED., 1944, **57**, 216.

§ According to Dr. Warren M. Cox, Jr., Director of Nutritional Research, Mead Johnson and Company, the thiamine comes largely from the barley malt (sprouted barley) used in the manufacture of dextri-maltose from cereal grains.

[7] Najjar, V. A., and Holt, L. E., Jr., *J. A. M. A.*, 1943, **123**, 683.

Summary. 1. Twelve female rats kept on a diet in which dextri-maltose constituted the sole source of nourishment survived on the average 85 days. 2. These rats lived 48 days longer than rats of the same weight kept on a single food diet of dextrose or sucrose; and 11 days longer than rats kept on dextrose with access to the 0.02 per cent solution of thiamine hydrochloride. 3. Their food intake was higher and they lost weight at a slower rate than the dextrose and thiamine rats. Their activity, water intake and vaginal smears were essentially the same. 4. It was concluded that the dextri-maltose contains sufficient amounts of thiamine to utilize to its fullest the available carbohydrate.

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[8] Najjar, V. A., *et al.*, *J. A. M. A.*, 1944, **126**, 357.

‖ Vitamin-free casein, Crisco, dextri-maltose, a mineral mixture and a vitamin mixture.

15052

Plasma Chloride and Bicarbonate after Potassium Administration.

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After nephrectomy, the plasma chloride of dogs falls gradually to low levels.[1] The anion is not lost to the exterior and must undergo redistribution. Since, after nephrectomy, potassium accumulates, it may carry chloride and bicarbonate as the potassium salt into tissue cells, such as muscle, according to the Boyle-Conway theory.[2,3] This situation has been mimicked over short periods of time by the injection of potassium nitrate dissolved in a minimum of water to avoid dilution of extracellular chloride and bicarbonate.

Methods. Mongrel male dogs given only water overnight were nephrectomized through the mid-ventral abdominal wall under intraperitoneal sodium amytal (55 mg of the acid per kilo). The potassium nitrate, 2.5 mM per kilo of dog, was injected intraperitoneally as a solution containing 500 mM KNO_3 per liter. Blood was collected from the femoral artery: before, and 2 hours, and 4 hours after the KNO_3 injection. Heparin was mixed into the blood which was collected under oil and covered with a rubber stopper before chilling and centrifugation.

Plasma was analyzed as in previous work[4] for water, dry residue (protein), chloride, and potassium. Sodium was weighed as the triple acetate in the Jena crucible according to Butler and Tuthill.[5] The bicarbonate as carbon dioxide of plasma was determined in duplicate, or in triplicate if checks were unsatisfactory, by the manometric method of van Slyke and Neill,[6] employing the minor modifications of Peters and van Slyke.[7] The pH of plasma was measured from whole

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[1] Atchley, D. W., and Benedict, E. M., *J. Biol. Chem.*, 1927, **73**, 1.

[2] Boyle, P. J., and Conway, E. J., *J. Physiol.*, 1941, **100**, 1.

[3] Wilde, W. S., *Bull. Math. Biophysics*, 1944, **6**, 105.

[4] Wilde, W. S., *Am. J. Physiol.*, 1945, **143**, 666.

[5] Butler, A. M., and Tuthill, E., *J. Biol. Chem.*, 1931, **93**, 171.

[6] van Slyke, D. D., and Neill, J. M., *J. Biol. Chem.*, 1924, **61**, 523.

[7] Peters, J. P., and van Slyke, D. D., *Quantitative Clinical Chemistry*, Methods, 283 *et seq.*, 292, Baltimore, 1932.

TABLE I.

Plasma Constituents in mM per Liter of Water; Actual Value Before (0) and Change During First 2 Hours (Δ_1) and During Second 2 Hours (Δ_2) After Peritoneal Injection of 500 mM KNO_3. Blood Cl values in mM per liter of blood.

Dog No.	Plasma Cl			Plasma K			Plasma Na			Blood Cl		
	0	Δ_1	Δ_2	0	Δ_1	Δ_2	0	Δ_1	Δ_2	0	Δ_1	Δ_2
1	116.8	—1.7		5.38	+4.60							
2	120.7	—3.5	—1.1	4.50	+6.52		161.5	—7.2				
3	121.5	—3.3	—0.7	4.04	+6.86	—0.58		(159.3)	—1.0	98.6	—13.9	+0.6
4	125.1	—2.4		4.57	+5.25					90.8	— 7.8	
5	119.7	—4.9	—0.6	5.09	+5.82	+0.79	159.0	—5.3	—2.2	91.5	— 6.0	—0.8
6	122.3	—3.0	—0.1	4.15	+7.03	+0.76	159.3	—6.8	+5.9	92.1	— 5.2	+0.8
7	127.0	—3.4	+0.6	5.00	+6.07	—0.59	162.4	—4.4	—0.4	95.7	— 7.6	—1.1
Mean		—3.17	—0.38		+6.03							
* σ		0.93	0.58		0.78							

* σ = Standard deviation.

blood drawn directly without anticoagulant into the micro chamber of the Coleman glass electrode. All concentrations are expressed in millimoles mM per liter of plasma water.

Results. The change Δ_1 in plasma chloride (Table I) during the first 2 hours after the potassium injection was only —3.17 mM. While by "t" test[8] the probability is less than 0.0014 that this represent a random variation, it is far below the change predicted mathematically[3] from Boyle-Conway. In fact to explain the small chloride decrement on such basis would require that the chloride of potassium enter cell water equal to only 17% of the body weight. Muscle water amounts to about 30% of body weight.

While the corresponding increment Δ_1 in potassium is of course significant statistically it bears no significant correlation[8] to the chloride decrement Δ_1 (r, 0.465).

Had plasma chloride and sodium been diluted together by some common mechanism (The sodium concentration also fell. Table I), chloride would have had the value Δ_1 of —4.6 instead of —3.17. In such a comparison we must remember of course that sodium bound as sodium proteinate in plasma can vary because of possible disturbance of capillary hydrostatic pressure relations in potassium poisoning or that some of the protein-bound Na can be released by ion exchange with the elevated K.

In preliminary experiments the insensible water loss as measured with an Ohaus balance for the 4 hours averaged 5 cc per kilo. This, as planned, is the quantity of water injected as 500 mM KNO_3. While the initial effect of any hypertonic solution placed in the peritoneal cavity is a dilution of extracellular electrolytes,[9] the final result with KNO_3, if, as is likely, it is completely diffusable,[4] is that the water injected as a solvent diffuses everywhere. Since this equals the insensible loss in volume we would expect no net change in electrolyte concentrations.

The peritoneal fluid at the end of 4 hours averaged 2.3 cc per kilo. This approximates the volume found normally after intraperitoneal amytal.

While the dry residue or protein of plasma varied in either direction the hematocrit ratio of erythrocytes in blood rose spectacularly (Table II) and continued to rise during the third and fourth hours. Since the water per unit dry weight of corpuscles did not change, the increased corpuscular volume represented hemoconcentration.

Though the hemoconcentration progressed into the second period, the electrolytes had reached equilibration in 2 hours as evidenced by the insignificant values of Δ_2 (Table I).

The possible exchange between injected nitrate and blood corpuscular chloride might be expected to raise the extracellular chloride. However in calculations of the concentration of chloride in corpuscular water in dogs 6

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[8] Treloar, A. E., *An Outline of Biometric Analysis*, t on p. 151, r on p. 171, Minneapolis, 1938.

[9] Darrow, D. C., and Yannet, H., *J. Clin. Inv.*, 1935, **14**, 266.

TABLE II.
Plasma Bicarbonate in mM per Liter of Water, pH of Plasma, Dry Residue of Plasma, and Hematocrit Ratio. Definition of 0, Δ_1, Δ_2 in Legend of Table I.

Dog No.	Plasma bicarbonate			Plasma pH			Dry residue of plasma, g/kg H_2O			Hematocrit, % erythrocytes		
	0	Δ_1	Δ_2	0	Δ_1	Δ_2	0	Δ_1	Δ_2	0	Δ_1	Δ_2
1	24.6	—0.6					75.5	+2.0		39.7	+ 5.7	
2	22.0	—2.2		7.38	0.00		79.6	—2.2		34.7	+ 0.3	
3	23.2	—2.5	—1.0	7.39	+0.11	+0.01	86.8	—1.6	—1.0	38.1	+11.9	+3.2
4	21.8	—7.2		7.40			82.5	+6.7		51.2	+12.2	
5	23.9	—5.4	—0.4	7.41	+0.09	+0.15	93.0	—1.3	—0.4	34.6	+ 6.2	+1.5
6	21.1	—0.6	—1.1	7.51	—0.03	—0.02	87.8	—4.0	—0.5	38.7	+ 4.0	+5.8
7	19.0	—4.5	—1.7	7.47	—0.01	—0.03	85.7	+6.4	—1.0	40.6	+ 8.1	+3.6

and 7, Δ_1 fell only 3.0 and 2.3 mM respectively. Since corpuscular water did not change we estimate that the chloride lost from the erythrocytes of these dogs could have raised the extracellular chloride 0.19 mM.

The sharp fall Δ_1 in the chloride of whole blood (Table I) is referable mainly to the increased proportion of corpuscles, which contain less chloride than plasma.

The plasma pH, measured in order to calculate bicarbonate and complicated by artificial respiration in dogs 5, 6, 7, is listed in Table II. The bicarbonate fell faster than chloride throughout, probably in relation to accumulating metabolic acids. However during the first 2 hours its value declined so much more than chloride in proportion to the normal concentrations of each as to suggest a special relation to the potassium administered. Whether the effect was related to a release of lactic acid by the potassium tetany or to some other factor remains unknown.

Summary. In nephrectomy a sudden experimental elevation of the extracellular potassium did not lower the plasma chloride as much as predicted from the Boyle-Conway concept. The chloride fell 3.17 mM while the potassium rose 6.03 mM and the correlation 0.465 between these is insignificant. The proportional fall in plasma sodium slightly exceeded that for chloride. The bicarbonate of plasma fell so rapidly initially as to suggest a special relation to the administered potassium.

15053

Shadowed Electron Micrographs of Bacteria.*

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In a recent note[1] we have described a procedure, based on metallic shadow-casting,[2] for enhancing the visibility of very small particles under the electron microscope and making apparent many details of their shapes which are not seen in other ways. This technic consists in obliquely depositing in a vacuum a thin layer of a relatively structureless metal such as chromium onto the electron microscopic preparation. The distribution of thickness of this layer, which should be of such an average thickness as to be partially transparent to the electron microscope beam, is determined by the contours of the preparation.

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* Supported in part by a grant from the National Foundation for Infantile Paralysis, Inc.

[1] Williams, R. C., and Wyckoff, R. W. G., PROC. SOC. EXP. BIOL. AND MED., 1945, **58**, 265.

[2] Williams, R. C., and Wyckoff, R. W. G., *J. Applied Physics*, 1944, **15**, 712.

Relatively tall objects collect a thicker deposit in the direction of the oncoming metal atoms and at the same time shield other areas to produce what might be called permanent "shadows" of themselves. As the previously published micrographs of influenza and tobacco mosaic virus particles[1] have shown, this results in a three-dimensional effect which brings out many details invisible by direct electron micrography. When this technic is applied to the photography of bacteria it proves to be equally useful for these larger objects. The present note describes this application and illustrates it with electron micrographs of several typical organisms.

Photographs of the following bacteria[†] are reproduced in the figures of this paper: (1) *Bacillus subtilis,* a recently isolated smooth strain; (2) *Bacillus mesentericus vulgatus,* a strain of the "Michigan" type cultivated for many years in the laboratory; (3) *Salmonella typhimurium,* a strain cultivated for 3 years in the laboratory; (4) *Bacterium typhosum,* an old laboratory strain; (5) *Staphylococcus aureus,* a strain isolated 3 years ago from human material; (6) *Streptococcus hemolyticus* (beta), scarlet fever strain IV (Dick); (7) *Streptococcus pneumoniæ,* Type II, an old laboratory strain.

Bacteria to be photographed were grown in broth or on agar slants, the cultures varying in age from 24 hours to 3 months. Organisms were washed from the slants; the resulting suspensions were made 0.5% with respect to formalin and then sedimented by centrifugation at 2000 r.p.m. Broth cultures were similarly formalinized and sedimented. In either case the sedimented cells were resuspended in distilled water and washed twice by alternate centrifugation and resuspension in water. Preparations for electron microscopy were made from such purified suspensions by adding micro-drops of appropriate dilutions to the surface of the usual collodion-covered metal screens. Films of metallic chromium of *ca.* 7 millimicron calculated average thickness were then evaporated obliquely onto the top surfaces of these preparations after they had thoroughly dried in the air. Metal evaporation was carried out as in the work with viruses except that for these large objects the angle of deposition was chosen so that shadow-lengths would be only twice the heights of the objects casting them.

Organisms[‡] in an old culture of *B. subtilis* appear as in Fig. 1 after being shadowed. The central protoplasm in the 3 bacilli lying side by side stand out as opaque masses. The neighboring, more transparent, convoluted mass from which a single flagellum seems to issue is perhaps the extruded protoplasm from a fourth disintegrated cell. An isolated *subtilis* bacillus is shown in Fig. 2a. This cell, like those of the preceding figure, has a relatively flat shelf of sharply bounded substance beyond its opaque central protoplasm; this probably outlines the cell wall from which the central mass has retracted on aging and drying. When this bacillus is photographed with

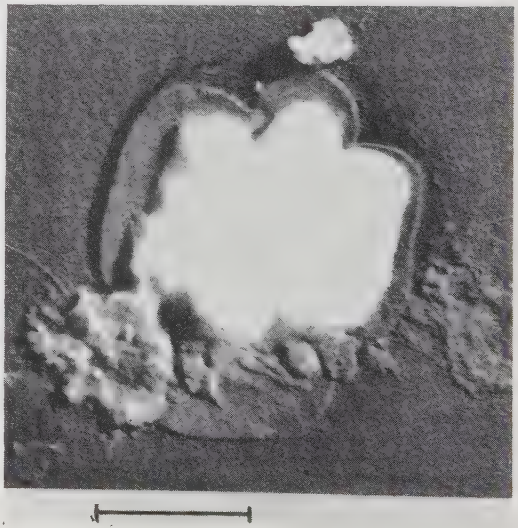

FIG. 1.

A shadowed electron micrograph of a group of *B. subtilis* organisms. The scale of magnification of this and the following photographs is given by the one micron scale appended to each. It shows that the magnification in these reproductions, except Fig. 4, is about 20,000 times.

[†] We are indebted to Dr. J. M. Bourn of the Michigan Department of Health Laboratories for bacterial cultures.

[‡] For excellent electron micrographs of subtilis and subtilis-like bacilli see for example Mudd, S., Polevitzky, K., Anderson, T. F., and Chambers, L. A., *J. Bact.*, 1942, **42**, 251; Dubin, I. N., and Sharp, D. G., *J. Bact.*, 1944, **48**, 313.

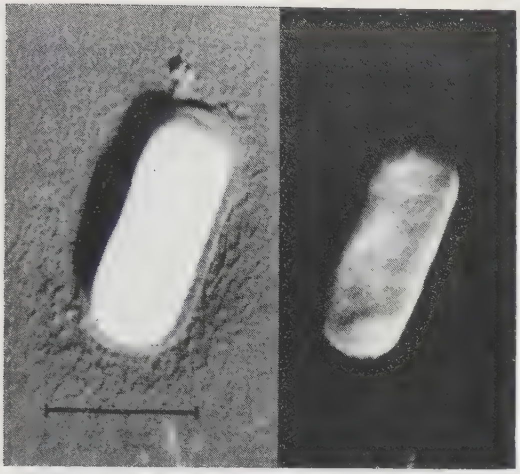

FIG. 2a (left).
A light exposure of a younger single cell of *B. subtilis* after metal-shadowing, showing the cell outline and wall and, by its "shadow," the general shape of the bacillus.

FIG. 2b (right).
A prolonged exposure of the same shadowed preparation showing the surface contours of the bacillus.

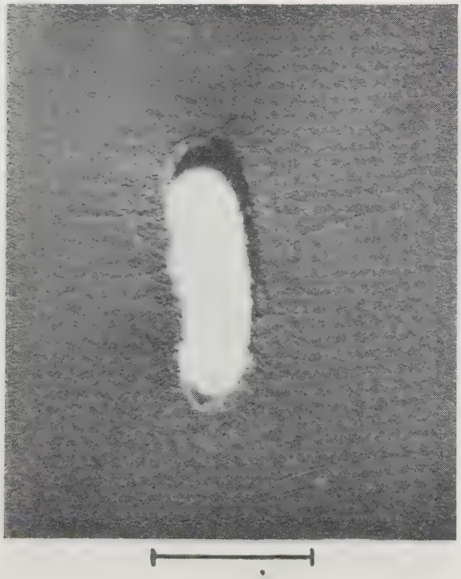

FIG. 3.
A shadowed micrograph of a single organism from a culture of *Bact. typhimurium*. The exceedingly fine peritrichate flagella of the cell are evident.

such a prolonged exposure that it appears partially transparent, Fig. 2b is obtained. This is primarily a record of the contours of its surface since the electron scattering of the deposited metal so greatly exceeds that of the bacterial protoplasm that it concentrates the observed scattering in this superficial layer. Other examples of the delineation of surface structure that can be made through such prolonged exposures are given in subsequent photographs. As would be expected, the related *B. mesentericus vulgatus* gives photographs much like those shown above for *B. subtilis*. Several old cells, all but one of which were undoubtedly dead before the preparation was made, are reproduced in Fig. 5. Fragments of extruded protoplasm and many of the very thin flagella possessed by these big rods are strewn over the field. The large spore at the bottom of this picture has structure which is reflected in its partial transparency to the electron beam.

Photographs of 2 flagellated members of the genus Bacterium are reproduced in Fig. 3 and 4. The single cell of *Bact. typhimurium* (Fig. 3) is almost entirely filled with its protoplasm but a narrow surrounding shelf is visible and beyond this the very fine peritrichate flagella are readily seen. The much

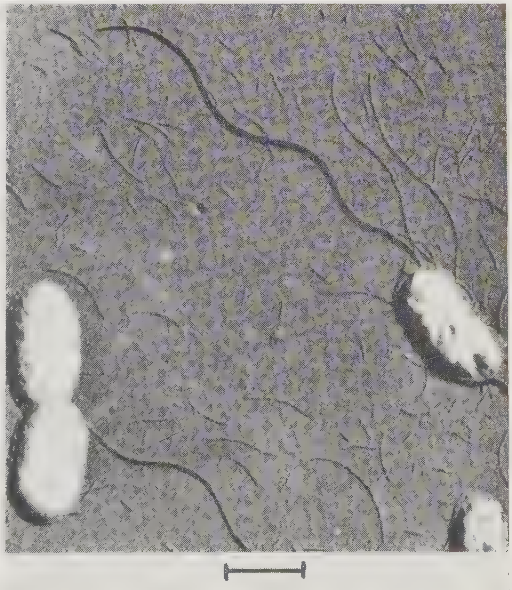

FIG. 4.
A shadowed micrograph of 3 cells of *Bact. typhosum* showing flagella. The magnification has been reduced to about half that of the other figures in order to include an entire flagellum. Note also the many fragments of finer flagella.

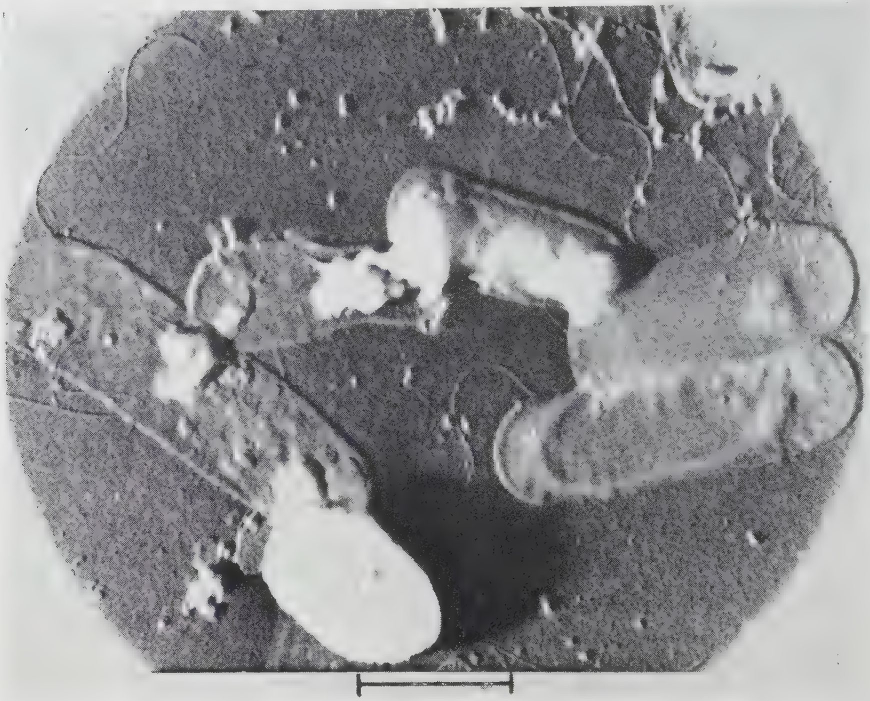

Fig. 5. A shadowed micrograph of several old cells and a spore from a culture of *B. mesentericus vulgatus.*

thicker flagella of some typhoid bacilli[3] are evident in Fig. 4. They cast shadows of very different lengths and must therefore vary greatly in thickness; often they appear more as ribbons than as threads.

The photographs that follow are of various cocci. The spherical streptococci of Fig. 6 are bound together by connections whose details can be clearly seen. Details of their surface as well as of the double cup-like structure, faintly apparent in the single coccus on the edge of this figure can be brought out by longer exposure. The wrinkled surfaces of the diplococci of a 24-hour culture of Type II pneumococci are evident in the prolonged exposure of Fig. 7. While it is possible with care to bring out slight indications of capsular substance surrounding such pneumococci, these indications are so slight as to defy satisfactory reproduction. Fig. 8 and 9 are examples of the pleomorphism seen in cultures of *S. aureus.* The surfaces of the cocci of both figures are at least as rough as those of the pneumococci. The white, especially opaque

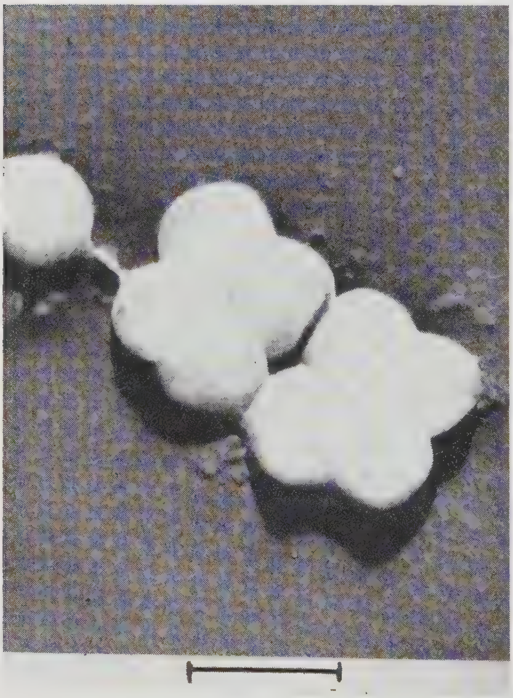

Fig. 6.
A shadowed micrograph of several cells from a culture of *Str. hemolyticus* IV (Dick).

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[3] Mudd, S., and Anderson, T. F., *J. Exp. Med.*, 1942, **76**, 103.

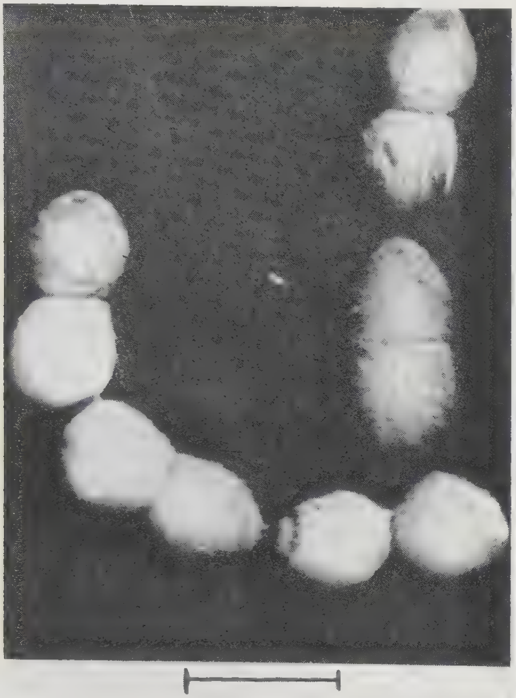

Fig. 7.
A prolonged exposure of a shadowed preparation of diplococci from a culture of *Str. pneumoniæ* II, showing the surface structure of these cells.

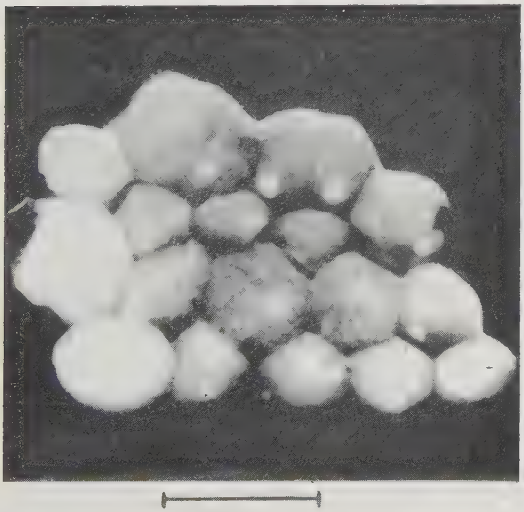

Fig. 8.
A prolonged exposure of a shadowed preparation of a group of organisms from a culture of *Staph. aureus.*

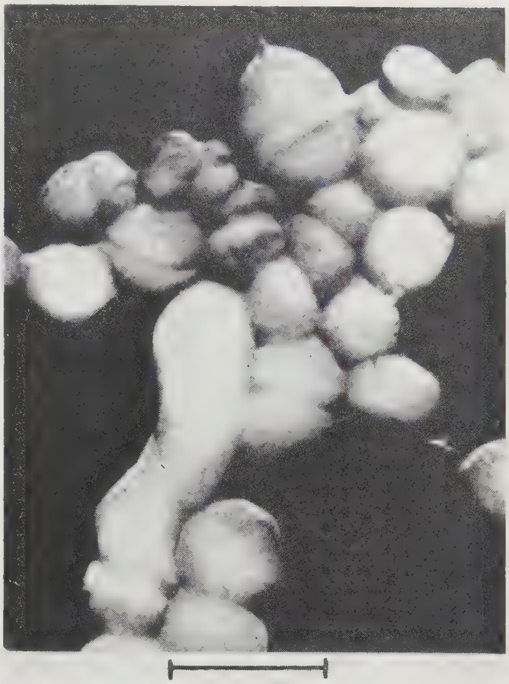

Fig. 9.
Another field from an old culture of *Staph. aureus* showing cells of various shapes.

regions in every cell of Fig. 8 are also present in unshadowed photographs.[4] Another structure often observed in these staphylococci is the broad equatorial band that is especially evident in the large form at the top of Fig. 9. Possibly it is an early stage of the division process that appears more developed in cells such as that at the center of this figure.

The foregoing micrographs illustrate the electron micrographic appearance of metal-shadowed bacteria. Our experience indicates that most information can be obtained by making two exposures of a shadowed preparation: (1) a light one to show the general shape of the organism, its flagellar structure, and any evidence there may be of a capsule or other substance surrounding the cell proper, and (2) a prolonged exposure which will bring out details of its surface.

Summary. Metal shadow-cast preparations reveal under electron microscopy many details of bacterial morphology. The three-dimensional effect achieved with this technic brings out with especial clarity the shapes of organisms and the contours of their surfaces; shadowing also makes evident their flagellar and other extra-cellular processes. Illustrative shadowed electron micrographs are given of subtilis-like and typhoid-like organisms and of several forms of cocci.

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[4] Knaysi, G., and Mudd, S., *J. Bact.*, 1943, **45**, 348.

15054 P

Cultivation of Viruses of the Psittacosis Group in the Allantoic Cavity of Chick Embryos.*

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Many of the viruses of the psittacosis group have been cultivated successfully in fertile hens' eggs, the yolk sac being most satisfactory for this purpose. Using this particularly susceptible tissue, preparation of large amounts of virus has been possible.[1] Such preparations cannot be regarded as ideal for some purposes, however, such as attempts at purification of the virus[2] or for production of antigens, because of the large amount of admixed tissue debris and yolk material, removable only with difficulty. It occurred to us that the allantois, being embryologically similar to the yolk sac, might also show special susceptibility to the viruses of this group. The allantoic cavity has been extremely useful in cultivation of influenza virus,[3] and the advantages in the case of the latter virus might be expected to hold for others as well.

After our first attempts to adapt mouse pneumonitis virus, one of the agents of this group, to the allantoic cavity, the publication of Williams[4] appeared, in which he reports successful cultivation of psittacosis virus in the allantoic cavity and in greater amounts than obtained in the yolk sac. We have been able to confirm his finding and have extended the study to other viruses of the group. The present preliminary report records the successful cultivation of a strain of psittacosis, human pneumonitis (SF), and meningopneumonitis (F-97) virus in the allantoic cavity. The few attempts made so far to adapt mouse pneumonitis virus to the allantoic cavity have not been successful. Williams reported failure to cultivate lymphogranuloma venereum virus in this tissue.

The strains of virus used in the present experiments have been employed in previous work in this laboratory and their sources have been recorded.[2] The first inoculations into allantoic cavities were made with emulsions of infected mouse tissue or yolk sac, and subsequent passages were made with 0.25 cc quantities of undiluted allantoic fluid. The preparations of human pneumonitis and feline pneumonitis virus, designed for the first allantoic injections, proved to be contaminated with bacteria, a circumstance overcome by adding sulfadiazine to the contaminated emulsions in the amounts of 68 to 75 mg per egg. Before use the eggs were incubated at 38 to 39°C for 7 or 8 days and at 37°C for 2 days. After the inoculations were made, incubation was continued at 35° C. When movements of the embryo became feeble, indicating impending death, usually on the 3rd, 4th or 5th day, the eggs were chilled at 4° C for a few hours and the allantoic fluids then withdrawn. Five to 6 cc per

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* This investigation has been supported by: The Commission on Influenza, Board for the Investigation and Control of Influenza and Other Epidemic Diseases in the Army, Preventive Medicine Service, Office of the Surgeon General, U. S. Army; The John Rockefeller McCormick Memorial Fund of the University of Chicago.

[1] Grace, A. W., Rake, G., and Shaffer, M. F., Proc. Soc. Exp. Biol. and Med., 1940, **45**, 259.

[2] Hilleman, M. R., *J. Infect. Dis.*, 1945, **76**, 96.

[3] Burnet, F. M., *Aust. J. Exp. Biol. and Med. Sci.*, 1941, **19**, 291.

[4] Williams, S. E., *Aust. J. Exp. Biol. and Med. Sci.*, 1944, **22**, 205.

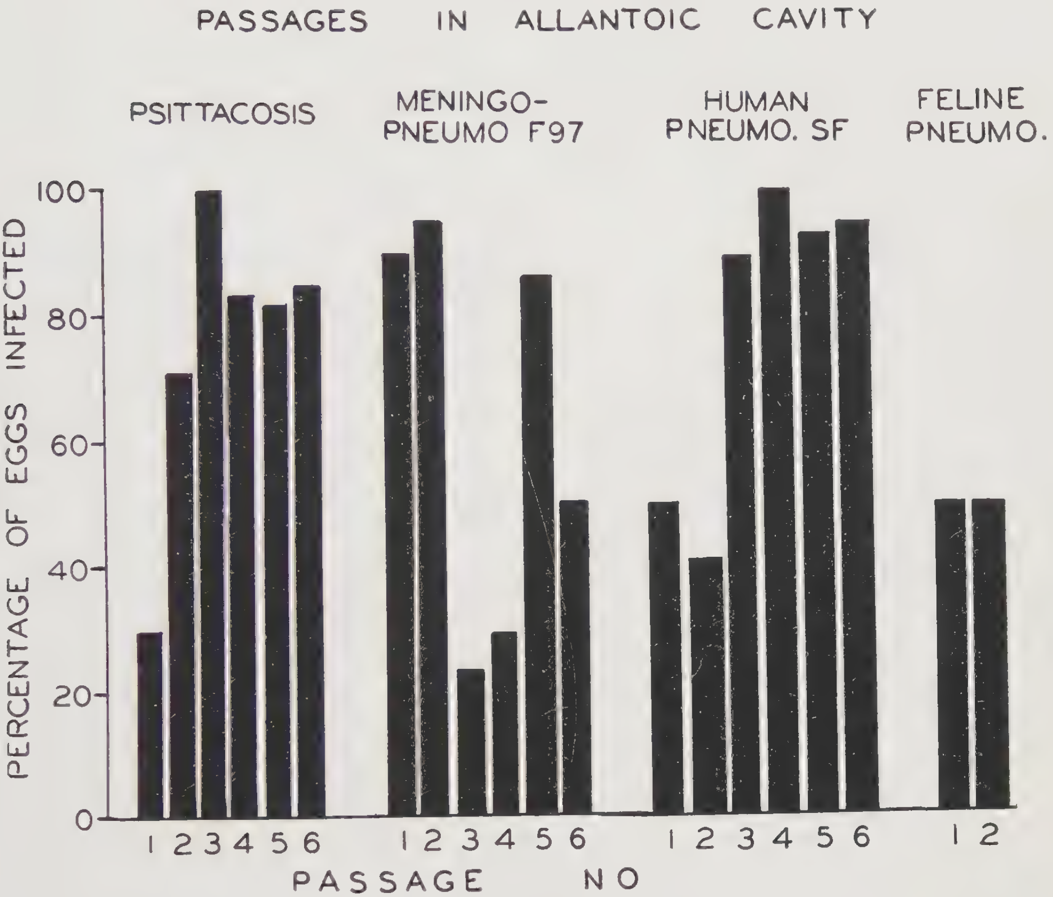

CHART 1.

Passages of Virus in the Allantoic Cavity of Fertile Eggs.

Each passage consisted of 6 to 60 eggs and infection was determined by stained smears of allantoic fluid.

TABLE I.

Titration of Allantoic Fluids by Mouse Inoculation.

Virus	Egg passage No.	Route of inoculation* of mice	Results in mice Representative mean infectivity scores† 10^{-3}	 10^{-4}	 10^{-5}	 LD$_{50}$
Psittacosis	3	intracerebral				$10^{-8.4}$
,,	6	,,				$10^{-9.2}$
Meningopneumonitis	3	intranasal	4.7	4.7	2.0	$10^{-4.4}$
,,	6	,,	5.0	4.3	2.3	$10^{-4.5}$

* Each inoculum consisted of a pool of 10 or more allantoic fluids which contained large amounts of elementary bodies as revealed by stained smears.

† Computed according to the method of Horsfall.[5]

egg were obtained. From 6 to 60 eggs were used for each passage and the determination whether an egg was infected was made by the presence or absence of elementary bodies in stained (Macchiavello) smears of the allantoic fluid. Chart 1 indicates the percentage of infected eggs at each passage and suggests that satisfactory adaptation to the allantoic cavity had occurred by the 3rd passage in the case of psittacosis and human pneumonitis virus. The reason for the irregular results with meningopneumonitis virus is unknown. In the 2 passages with feline pneumonitis virus completed to date only one half the eggs became infected, results which are similar to those of 2 of the other agents.

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[5] Horsfall, F. L., *J. Exp. Med.*, 1939, **70**, 209.

Bacteria-free allantoic fluids of a single passage were pooled in groups of 10 or more, only those fluids being chosen in which stained smears had revealed large numbers of elementary bodies. Several of these pools have been titrated by mouse inoculation, the results of which are given in Table I. The titers indicate concentrations of virus in these preparations which compare favorably with preparations from other sources. For example, an emulsion of yolk sac tissue infected with meningopneumonitis virus, when titered in mouse lung, gave infectivity scores[5] of the same range as those indicated in Table I for allantoic fluid, 5.0, 4.3 and 2.6 for the 10^{-3}, 10^{-4} and 10^{-5} dilutions respectively.[6] The LD_{50} value of the meningopneumonitis virus[†] from yolk sac was $10^{-4.3}$; the comparable values for infected allantoic fluids were $10^{-4.4}$ and $10^{-4.5}$ (Table I). Our strain of psittacosis virus, when propagated in mouse brain, gave an LD_{50} in mice[7] of $10^{-4.5}$. The comparable figure for allantoic fluid (*e.g.*, LD_{50} equals $10^{-8.4}$) indicates approximately a 10,000-fold difference in favor of the allantoic fluid. This figure confirms closely that reported by Williams.[4]

Judging by the numbers of elementary bodies in stained smears, large amounts of virus are present also in the allantoic fluids infected with human pneumonitis but only small amounts were present in the first 2 passages of feline pneumonitis virus Quantitative determinations by mouse titration have not yet been completed in the case of these 2 latter viruses.

Preliminary attempts at concentration of psittacosis elementary bodies from allantoic fluid indicate that this is easily accomplished by centrifugation, with little difficulty arising from the presence of tissue debris.

These studies suggest that growth in the allantoic cavity of fertile eggs may offer certain advantages over previous methods of cultivation, in the case of some members of the psittacosis group of viruses. The infected allantoic fluid represents a ready-prepared suspension of virus in high concentration, available in large amounts, with relatively little extraneous matter present.

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[6] We are indebted to Elizabeth St. John for these figures from unpublished work.

[†] A personal communication from Dr. Thomas Francis, Jr., states that the allantoic route of inoculation has been used for some time in his laboratory for propagation of meningopneumonitis virus. The allantoic fluid, rich in elementary bodies, proved satisfactory as antigen in the complement fixation test, and the infective titer was as good or better than that of similar quantities of mouse lung.

[7] Calculations made by method of Reed, L. J., and Muench, H., *Am. J. Hygiene*, 1938, **27**, 493.

15055

Aggressin-Like Character of Gastric Mucin.

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Since Nungester, Wolf, and Jourdonais[1] reported that gastric mucin increases the virulence of meningococci, streptococci, and staphylococci, when injected simultaneously into the abdominal cavity, this technique has been used to reinforce the virulence of gonococci,[2] *H. influenzae*,[3] typhoid organisms,[4] and *H. pertussis*.[5] Mucin suspensions of such microorganisms, otherwise not suited for experimental infection, facilitate their use for determining on a quantitative basis the potency of chemotherapeutic agents, vaccines, and antisera. Staphylococcus[6] and meningococcus infections with mucin are used in our laboratory routinely for studying the activity of chemotherapeutic agents. More recently, we succeeded by the use of the same technique in obtaining a lethal infection with *C. diphtheriae* in mice. This successful infection gave us the opportunity to study the antibacterial activity of certain chemotherapeutic agents against *C. diphtheriae*.[7]

The mode of action of mucin has found various interpretations. It seems, though, that the question is far from settled.

Miller and Castles[8] consider that mucin may interfere locally with the defense mechanism of the host, thereby making the barrier between peritoneal cavity and blood stream permeable for meningococci. According to Nungester, Jourdonais, and Wolf,[9] mucin inhibits the bactericidal properties of phagocytic cells without influencing phagocytosis. Keefer and Spink[10] reported that mucin depresses the bacteriolytic power of serum for gonococci (without lowering the complement titer) by somehow protecting the organisms against the action of natural antibodies. McLeod[11] suggests that mucin acts mainly as a favorable medium for the propagation of meningococci in the peritoneal cavity from where the blood stream is then progressively invaded. The function of mucin as a medium favoring growth is in contrast with previous observations made by Miller and Castles for meningococci[8] and by Keefer and Spink for gonococci.[10] The literature on this subject has been reviewed by McLeod.

In the course of our studies on staphylococcal and diphtherial infections, a number of observations have been made which may contribute towards the elucidation of the mechanism of mucin activity.

Material and Method. The preparation and standardization of the inocula and the technique used for infection have been described before.[6,7] Granular mucin Type 1701-W, batches No. 48555 and 52012 of the Wilson Laboratories, was used throughout the investigation. *Staphylococcus aureus* 6340, a strain originally obtained from an infected patient and *C. diphtheriae* No. 9060, type gravis, used in these experiments were received from the American Type Culture Col-

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[1] Nungester, W. J., Wolf, A. A., and Jourdonais, L. F., Proc. Soc. Exp. Biol. and Med., 1932, **30**, 10.

[2] Cohn, A., and Peizer, L. R., *J. Inf. Dis.*, 1938, **63**, 77.

[3] Fothergill, L. D., Dingle, J. H., and Chandler, C. A., *J. Exp. Med.*, 1937, **65**, 721.

[4] Rake, G., Proc. Soc. Exp. Biol. and Med., 1935, **32**, 1523.

[5] Silverthorne, N., *Canad. Pub. Health J.*, 1938, **29**, 233.

[6] Ercoli, N., Lewis, M. N., and Harker, E. M., *J. Bact.*, 1944, **47**, 451; *Am. J. Med. Sciences*, 1945, **209**, 621.

[7] Ercoli, N., Lewis, M. N., and Moench, L. J., *J. Pharmacol. and Exp. Therap.*, in press.

[8] Miller, C. P., and Castles, R., *J. Inf. Dis.*, 1936, **58**, 263.

[9] Nungester, W. J., Jourdonais, L. F., and Wolf, A. H., *J. Inf. Dis.*, 1936, **59**, 11.

[10] Keefer, C. S., and Spink, W. W., *J. Clin. Invest.*, 1938, **17**, 23.

[11] McLeod, Charlotte, *Am. J. Hygiene*, 1941, **34**, 41, 51.

TABLE I.
Leucocytosis and Destruction of the Staphylococci Injected in Mucin and in Saline.

Infection		Staphylococci in the culture of the peritoneal exudate after hours					Leucocytosis in peritoneal smear after hours	
No. of staphylococci	Vehicle	2½	3½	5	7½	25	7½	25
71,600	saline	±	±	±	0	0	xxx	xxx
71,600	”	0	±	±	±	0		
71,600	mucin	++	+	++	+++	+++*	*x*	x
7,160	”	++	+	+	+	++	*x*	xxx

0 No growth.
± 2-20 colonies.
+, ++, +++ Different intensity of growth.
* Died 4 hours later.
x A few cells.
x, xxx Different intensity of leucocytosis.

lection. The meningococcus Group I strain was received from Dr. E. A. Kabat, College of Physicians and Surgeons, Columbia University.

(1) *Fate of injected cocci.* (*a*) *In the peritoneal cavity.* Cultures of peritoneal fluid made on agar plates revealed that staphylococci injected alone in saline readily disappear from the peritoneal cavity. During the first 2½ to 5 hours following inoculation, only occasional colonies grew from the exudate. No growth was obtained in the cultures made from the exudate 10 to 24 hours after inoculation.

Staphylococci injected into the peritoneal cavity with mucin remained viable. Cultures of peritoneal fluid made 2½ to 5 hours after inoculation gave on the agar plate a more or less confluent growth of staphylococcus. The number of colonies increased with the time elapsed after inoculation. The experiment reported in Table I shows that 7000 staphylococci injected in mucin gave a confluent growth from peritoneal liquid 25 hours after inoculation, while 10-fold heavier inocula injected without mucin could not be detected in cultures made after 7½ hours.

(*b*) *Spreading of staphylococci with and without mucin from the peritoneal cavity.* The spreading of cocci from the site of infection after injection in mucin was traced by culturing blood from the tail vein of living mice and by culturing heart and lung tissue of animals killed after different intervals. Heart and lung cultures showed staphylococci ½ to 1 hour after intraperitoneal inoculation with 30,000 to 50,000 cells; growth became particularly abundant after 2 to 3 hours. (Peripheral blood cultures showed the presence of occasional staphylococci during the first 10 hours after inoculation, but the number of organisms definitely increased only after 18 to 24 hours when the mice were beginning to die.) No bacteriemia was observed if the same dose of staphylococci (30,000 to 50,000) was suspended in broth and injected intraperitoneally. Blood-agar plate cultures from lungs and heart, taken at frequent intervals during the first 24 hours, were negative. The cultures from organs remained negative even after increasing the number of injected staphylococci to one million. A typical experiment is given in Table II.

These results suggest that the addition of mucin was responsible for the spreading of the organisms from the site of infection into the organs.

2. *Reactions in the Peritoneal Cavity.* Peritoneal fluid of mice injected with 30,000 to 70,000 staphylococci (with and without mucin) was collected at various intervals after inoculation by puncturing the abdominal wall with a capillary pipette. The fate of the injected cocci and the resulting inflammatory reaction were followed by making smears stained with methylene blue.

Mice injected with 50,000 staphylococci suspended in saline showed a strong leucocytic reaction 2½ to 5 hours after inoculation. On microscopic observations of the stained smears neither extra- nor intracellular cocci could be seen. *A similar inoculum injected with mucin showed a strikingly different picture.* Only isolated leucocytes, if any, appeared and, in many instances, their shape was irregular. While the microscopic observations of stained

TABLE II.
Invasiveness of Staphylococci Injected Intraperitoneally in Mucin and in Broth.

No. of staphylococci injected, × 1000	Interval after inoculation, hr	Infection with mucin		Infection without mucin	
		Heart Culture	Lung Culture	Heart Culture	Lung Culture
26 to	½ to	0	0		
135	1½	4 colonies	±		
		1 colony	3 colonies	0	0
		+	+	0	0
		+	—		
		±	±		
	3½ to	+*	+*	0	0
	7½				
	12½	+	+		
		++	++	0	0
	24	+++†	+++	0	0
1,350	4			0	0
	6			0	0

0 No growth.
± Non-confluent growth.
+ Confluent growth.

* 8 mice.
† 6 mice died.

smears taken between 3 to 8 hours after inoculation failed to reveal the presence of cocci in the peritoneal exudate, we know from the results obtained by culturing this exudate that viable cocci were present.

It seems that the effect of mucin on the appearance of peritoneal fluid is two-fold: (1) inhibition of leucocytosis; (2) lack of destruction of cocci. The most plausible interpretation is that staphylococci in mucin survive in the peritoneal fluid, because they are not destroyed by leucocytes which normally would have appeared in large number.

3. *Heated Staphylococci.* For a further analysis of the effects of mucin, heat-killed staphylococci were used. A heavy suspension of cells was heated for 2 to 3 hours at 60°C. (In some experiments, this treatment failed to kill all the cocci.)

One hundred million of dead staphylococci injected intraäbdominally in 0.5 cc saline caused a strong leucocytic reaction 4 hours after inoculation. As shown by smears, the leucocyte count remained high for 24 hours. With the same amount of vaccine injected in 0.5 cc of mucin, only a few leucocytes were found on the slide 4 hours after inoculation. After 6½ hours, again only a few leucocytes, most of them containing intracellular cocci, were visible.

Thus, mucin exerted the same influence on the disposal of heat-killed as on that of live staphylococci. Death from bacteriemia resulted when mice were given mucin with the vaccine which still contained some live cocci, since as stated earlier, exposure of the bacterial suspensions to heat at 60°C for 2 to 3 hours did not result in killing all the organisms. Staphylococci could actually be cultured from the heart and other organs of these animals.

4. *Other Infections.* (*a*) *Meningococcus Group I.* A limited number of experiments gave results similar to those described for staphylococcus. A characteristic experiment is given in Table III. It would appear that during the first stage following inoculation, mucin inhibits leucocytosis and, consequently, the destruction of meningococci.

(b) *C. diphtheriae.* The fate of the bacillus was followed in the peritoneal cavity and in the organs of mice infected with and without mucin by making cultures on solid media containing tellurite. The results are given in Table IV and indicate that (1) mucin inhibits local leucocytosis after intra-abdominal injection of *C. diphtheriae;* (2) the presence of mucin interferes with the destruction of diphtheriae bacilli in the peritoneal cavity, while in the absence of mucin, such destruction takes place within 2½ to 5 hours; (3) mucin enhances the spreading of the diphtheriae infection from the peritoneal cavity.

TABLE III.

Influence of Mucin on the Intraäbdominal Infection of Mice with Meningococci (Group 1).

Mouse No.	Slant suspension diluted	Suspended in (1 cc)	1½ hr after infection: Culture of† peritoneal fluid	1½ hr after infection: Culture of† heart	1½ hr after infection: Local leucocytosis	3 hr after infection: Culture of peritoneal fluid	3 hr after infection: Culture of heart	3 hr after infection: Local leucocytosis
15647	10^{-4}	mucin*	++	1 colony	0			
648	,,	,,			0	++	1 colony	0
673	,,	saline	±	0	+			
674	,,	,,				+	0	+++
670	10^{-5}	mucin	+	1 colony	0			
671	,,	,,			0	++	2 colonies	±
676	,,	saline	0	0	+			
677	,,	,,			±	4 colonies	0	++

* 100 MLD.

† On tryptose phosphate agar.

5. Effect of Mucin on Aleuronat Injected Intra-Abdominally. In order to investigate whether the leucocytosis-inhibiting activity of mucin is limited to bacteria, analogous experiments were made with aleuronat.

Two sets of experiments were carried out. In the first, aleuronat suspended in mucin was injected intra-abdominally; in the second, the effect of mucin was studied on mice previously treated with aleuronat.

Mice injected with a 1% aleuronat suspension in 1 cc mucin failed to show the usual leucocytic reaction within the first 2 to 6 hours; after 18 hours, no difference was observed between the effect of aleuronat in mucin and in saline.

One cc mucin injected into the peritoneal cavity of mice treated 18 hours before with aleuronat did not produce any noticeable destruction or diminution of leucocytes.

6. Mucin Antagonists: Aleuronat and staphylococcus infection with mucin.

From the previous experiments, one could draw the conclusion that mucin exerts its claimed virulence-increasing effect by inhibiting leucocytosis. However, since an already existing leucocytosis in the peritoneal cavity is not influenced by mucin, we were able to study the mucin effect in the presence of leucocytes.

Local leucocytosis was induced by aleuronat. Subsequently, a staphylococcus infection with mucin was given. It was expected that the mucin effect in the presence of a leucocyte infiltration would not take place. The data given in Table V confirmed this expectation.

Cultures made of peritoneal fluid 3 and 5½ hours after inoculation gave a confluent growth for the untreated mice and only a small number of colonies (35-55) for mice previously treated with aleuronat. Thus, in the presence of leucocytes, the destruction of staphylococci takes place rapidly even if they had been injected with mucin. In other words, the activity of mucin is neutralized by leucocytes accumulating after a previous aleuronat injection.

No protection against bacteriemia could be obtained with aleuronat if a more strongly invasive coccus, such as *Streptococcus hemo-*

TABLE IV.

Influence of Mucin on the Intraäbdominal Infection of Mice with *C. diphtheriæ*, Type Gravis, No. 9060.

Mouse No.	Broth diluted	in (1 cc)	2 hr after infection		3 hr after infection			5 hr after infection		
			Cultures peritoneal fluid	Local leucocytosis	Cultures of peritoneal fluid	Cultures of heart	Local leucocytosis	Cultures of peritoneal fluid	Cultures of heart	Local leucocytosis
11,189	10-2	mucin	+		++	±	±			
190	”	”	±							
191	”	”	+					+	±	±
195	”	saline	15 col.	±	10 col.	0	+			
196	”	”	3 ”							
197	”	”	15 ”	±				+	0	+++
192	10-3	mucin	±	±	+	0	0			
193	”	”	+	±						
194	”	”	±	±				+	15 col.	±
10,894	”	”	+	0	++		0	++	0	+
895	”	”	+	±	++		±	++	6 col.	0
933	”	saline	0		0			0	0	++
934	”	”	0	±	0		++	10 col.	0	

± = 1-2 leucocytes per oil immersion field.

lyticus, was injected. The virulence of the β-hemolytic streptococcus 4 (Group A, Type 3) was the same in the controls as in aleuronat pre-treated mice. This may have been due to the fact that even an increased leucocytosis is not sufficient to destroy completely such a virulent organism. The observation would be in agreement with the data reported by Menkin,[12] by Dennis and Berberian,[13] and by Schnitzer and Goddard.[14]

Discussion. The results obtained with live and heated staphylococci, with *C. diphtheriae*, meningococci, and aleuronat injected intra-abdominally suspended in mucin showed marked similarities. In every instance, it appeared that mucin inhibits local leucocytosis in the peritoneal cavity, increasing by this inhibition the invasive power of the injected organism. This observation is in agreement with the findings of Miller and Castles, indicating that cellular elements did not begin to accumulate in the peritoneal cavity until 3 hours after inoculation of meningococci and did not become abundant until the 9th hour.

When leucocytosis was already present, mucin did not seem to interfere with it: this means that leucocytes were not destroyed by mucin; consequently, the increase of virulence was slight when staphylococci plus mucin were injected into the already affected (by aleuronat) peritoneal cavity.

Thus, mucin may be considered an agent with negative chemotaxis and a direct antagonist to substances with positive chemotaxis, such as aleuronat, peptone, etc. Due to its property of negative chemotaxis, mucin decreases the resistance of the host towards bacteria even when their invasive power is low. In terms of classical immunology, one might say that mucin takes the place of the aggressin which is lacking in such microorganisms. As "aggressin substitute," mucin lowers the defensive powers of the host by preventing leuco- and phagocytosis, thus facilitating the spreading of the organisms and the invasion of the blood stream. In the case of diphtheria, for instance, the local defensive mechanism of the peritoneal cavity, which according to Seligmann and Jungeblut[15] "functions perfectly to ward off bacterial invasion and to maintain sterility," is abolished by the aggressin action of injected mucin.

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[12] Menkin, V., *J. Exp. Med.*, 1933, **57**, 977.

[13] Dennis, E. W., and Berberian, D., *Ibid.*, 1934, **60**, 581.

[14] Schnitzer, R. J., and Goddard, I. G., *J. Immunol.*, 1943, **46**, 133.

[15] Seligmann, E., and Jungeblut, C. W., *J. Immunol.*, 1941, **40**, 119.

TABLE V.
Antagonistic Effect of Aleuronat on Virulence of Staphylococci Induced by Mucin.

		Staphylococcus Infection + Mucin			
		Without aleuronat		With aleuronat	
Date of exp.	No. of staphylococci injected × 100	No. of mice	Survivors	No. of mice	Survivors
3/3/44	61	10	0	4	2
	610	10	0	5	4
5/10/44	65	5	1		
	650	10	4	5	4

Summary. 1. Virulent staphylococci injected in mucin do not produce a characteristic leucocytic reaction in the peritoneal cavity. 2. Staphylococci injected intraperitoneally in mucin remain viable and spread from the abdominal cavity; without mucin, they are rapidly destroyed. 3. Staphylococcus vaccine suspended in mucin does not produce leucocytosis during the first hours after injection. 4. *C. diphtheriae* and meningococcus injected intra-abdominally are influenced in the same manner by the presence of mucin. 5. The leucocytic reaction of aleuronat is inhibited by simultaneous injection with mucin. A later injection of mucin does not destroy leucocytes already present in the peritoneal fluid. 6. The virulence-enhancing effect of mucin can be neutralized by leucocytosis previously induced by aleuronat. 7. All these observations suggest that mucin decreases the resistance of the host to bacterial invasion and imparts to microörganisms of otherwise low virulence the potentialities of highly invasive microbes.

15056

Size of Histamine Wheal in Relation to Dose and to Animal Species.*

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Since the first description of the effects of histamine on the skin, there have been many studies and applications of this local reaction. This investigation concerns the variations in response to intradermal injections of histamine in different species, and also the relation of wheal diameter to the concentration of histamine used.

Eppinger[1] first described histamine urticaria produced by scarification. Sollman and Pilcher[2] obtained wheals with histamine by intradermal injection in man, but not in rabbits or cats. They used semi-quantitative methods to measure this skin reaction, finding that the size of the wheal produced by local administration appeared to be a function of the concentration of histamine used. A minimum reaction was obtained with a concentration of 1:100,000. Since this work, there have been numerous methods devised for the determination and assay of histamine, such as the uterine strip, patch test and chemical methods. However, for rapid

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* This investigation was undertaken with the aid of the staff of the Department of Pharmacology of the University of Southern California School of Medicine, Los Angeles, California.

[1] Eppinger, H., *Wien. med. Wchnschr.*, 1914, **113**, 1913.

[2] Sollman, T., and Pilcher, J. D., *J. Pharm. and Exp. Therap.*, 1917, **9**, 309.

routine analysis these leave much to be desired. In order that biological fluids might be assayed for histamine-like substances, the quantitative variation in wheal size with graded concentrations of histamine was studied. Finally, by use of this method of determination, histamine in the presence of a body fluid was measured.

Method. In order to determine the difference in the local skin reaction of man and various laboratory animals, histamine diphosphate† was injected intradermally into the shaved skin of a guinea pig, rabbit, cat, dog, goat (kid), and man. Three injections consisting of 1/20 cc of 1.0%, 0.1%, and a distilled water control were given to each. The resultant wheals were observed 20 min later, at which time the maximum response occurred. Wheals produced by distilled water were recorded as 0 and larger wheals were rated from + to ++++.

The relationship between wheal size and histamine concentration was determined in the following manner. A series of solutions of histamine diphosphate, 1.0, 0.1, 0.01, 0.001 and 0.0001%, and a distilled water control were injected intradermally into the shaved flanks of recently weaned kids. Two animals were used, series B and C being performed on the same animal with 2 weeks intervening. The maximum wheal diameters were measured after 20 minutes.

The following experiment was designed to show the effect of various concentrations of histamine in the presence of a biological fluid which itself either contained histamine-like substances (H-substance[3]) or some other irritative material. A series of histamine diphosphate solutions similar to that described earlier was made using, in place of distilled water, physiological saline mixed with an equal quantity of the intestinal fluid of a fasted cat. These solutions were injected intradermally in a kid and the resultant wheals were measured after 20 min.

TABLE I.
Whealing Produced by Intradermal Injections of Histamine in Various Animals.

	Histamine diphosphate 1.0%	Histamine diphosphate 0.1%	Distilled water control
Guinea pig	0	0	0
Rabbit	0	0	0
Cat	0	0	0
Dog	++++	+++	0
Goat	++++	+++	0
Man	+++	++	0

Results and Conclusions. It was found that the various animals tested could be divided into 2 groups: those which showed a marked whealing in response to intradermal injections of histamine, and those which showed only a minimum of traumatic response but no true histamine wheal. Table I. summarizes these data. These results are consistent with Dale's[4] observations that certain animals such as rodents respond to histamine by vasoconstriction while others such as the dog, monkey, and man, by vasodilation. The cat seems to occupy an intermediate position in that it shows a decrease of blood pressure[5] following intravenous histamine injection but no skin whealing following intradermal administration.

The diameters of wheals produced by intradermal injection in the kid were found to vary directly, within certain limits, with the concentration of histamine as shown by the solid line in Fig. 1.

Below 0.001% the concentration of histamine no longer determined the wheal diameter which was apparently dependent upon some other factor due to trauma, possibly the release of H-substance. Thus injection of 0.0001% histamine elicits a wheal approximately equal in size to one produced by the water control. This provides an interesting correlation with the work of Harris[6] who found by chemical analysis of normal skin an H-substance concentration of 0.0001%. Of note, also, is the fact that the variation in wheal diameter in the lower concentrations is far greater than in the higher concentrations. This can be explained on

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† Obtained from the Pfanstiehl Chemical Company.

[3] Best, C. H., and McHenry, E. W., *Physiol. Rev.*, 1931, **11**, 371.

[4] Dale, H. H., *Lancet*, 1929, **1**, 1179, 1233, 1285.

[5] Dale, H. H., and Laidlaw, P. P., *J. Physiol.*, 1910, **41**, 318.

[6] Harris, K. E., *Heart*, 1927, **14**, 161.

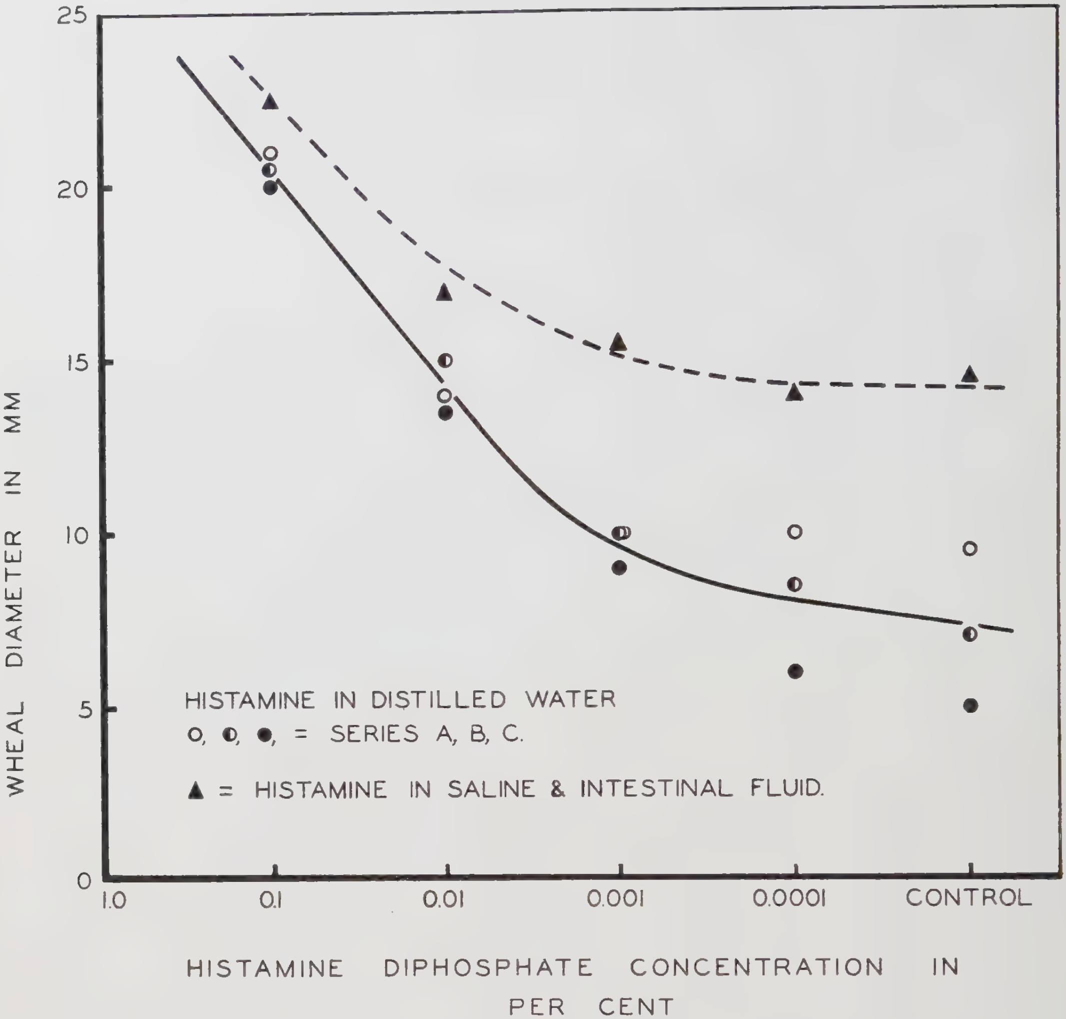

FIG. 1.

The relation of wheal diameter to concentration of histamine diphosphate injected intradermally in the goat. Solid line represents histamine in distilled water; broken line, in a mixture of physiological saline and intestinal fluid from a fasted cat.

the basis of uncontrolled factors causing variation of response to trauma incident to the injection, where histamine is no longer a limiting factor.

Measurement of histamine in the presence of a substance which also produces whealing is feasible providing the concentration of histamine is the limiting factor. Whereas the threshold with histamine alone is 0.001%, in the presence of a histamine-like substance, it is raised to 0.01% as shown by the broken line in Fig. 1. As the histamine diphosphate concentration increases, and the relative amount of H-substance decreases, the size of the wheal becomes increasingly dependent on the former, the 2 curves tending to converge.

These experiments suggest the importance of proper selection of laboratory animals for all dermatological investigations involving a local skin reaction. The response to histamine diphosphate injected intradermally provides a means of selecting animals which have a peripheral vascular mechanism similar to that existing in man. The use of dogs or goats would seem to be more logical than the time worn reliance on guinea pigs and rabbits for the study of response to local irritants and allergenic substances. The quantitative relationship existing between histamine concentration and skin wheal size suggests the possibility of using histamine as a standard reference material in comparing various skin irritants.

Summary. There is a species variation in the local reaction to intradermal injections of histamine. The dog, goat, and man show a marked skin whealing, but no such response is obtainable in the guinea pig, rabbit, or cat. The diameter of the skin wheal produced by intradermal injection is a function of the concentration of histamine used, the threshold being 0.001%. In the presence of an extraneous histamine-like substance, this relationship still exists, but the threshold of histamine sensitivity is elevated to 0.01%.

A practical application to the field of experimental dermatology is suggested involving the proper selection of test animals and the use of histamine as a standardizing agent.

15057

Ulcer Production in Intestines of Dogs by Various Enzymes Under Hydrostatic Pressure.

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In a previous paper from this laboratory the effect of hydrostatic pressure on the production of ulcers was described.[1] In another paper it was reported that ulcers could be produced in the intestines of dogs by an alkaline solution of pancreatin under a hydrostatic pressure of 90 cm H_2O.[2]

The purpose of the experiments reported here was to study the effect produced on the intestines by the following enzymes: rennin, trypsin, erepsin, steapsin, and amylopsin. The enzyme solutions were at pH ranges corresponding to those under which the enzymes act in the normal animal.

The procedure was the same as that described in [1] except that in these experiments only one pressure (90 cm H_2O) was used. The severity of damage to the loops was graded according to a scheme adopted in this laboratory which is described here: (1) represents a condition in which there is a slight surface necrosis of the mucosa; (2) designates a deeper necrosis involving at least ⅓ of the thickness of the mucosa of the entire area with smaller areas (from ½ cm^2 to 2 cm^2) showing necrosis as deep as the submucosa; (3) indicates necrosis of practically all the mucosa with some patches extending into the circular muscle; (4) was applied to a loop having areas of necrosis extending to the serous coat or to a frank perforation. Each loop was scored in this manner and the average taken for each group of experiments. The results are given in Table I.

Discussion. Rennin (0.1%) in N/10 HCl produced perforating ulcers in 114 minutes while N/10 HCl alone caused perforation in 174 minutes and pepsin (0.1%) in N/10 HCl in 82 minutes.[2] These data show that rennin has a marked digestive effect on the mucosa of the intestine but not so great an effect as pepsin.

Of the other enzymes studied only the proteolytic ones were active. The necrosis produced by trypsin was about twice as severe as that caused by erepsin, but not even trypsin digested the mucosa to an extent comparable to that of rennin and pepsin, and was only about one-half as effective as pancreatin.[2]

Neither steapsin nor amylopsin caused any necrosis of the mucosa, alkaline solutions of these enzymes behaving like controls where the loops of intestine were exposed to the

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[1] Driver, R. L., Chappell, R. H., and Carmichael, E. B., *Am. J. Dig. Dis.*, 1945, **12**, 168.

[2] Driver, R. L., *Arch. Path.*, in press.

TABLE I.
Ulcer Formation in Intestines by Enzyme Solutions under 90 cm H_2O Pressure.

Dog No.	0.1% solution of	pH	Time exposed (min.)	Remarks	Severity
1-12	rennin* in N/10 HCl	1.2	60-180	perforated ulcer in all dogs	4.0 in all dogs
13	trypsin† in N/10 $NaHCO_3$	8.1	580	necrosis through ½ thickness of mucosa entire area	2.0
14	” ” ” ”	”	698	necrosis through ⅓ thickness of mucosa over entire area, 10 small spots into submucosa	2.0
15-17	” ” ” ”	”	326-384	normal mucosa	0
18	” ” ” ”	”	696	necrosis through ⅓ thickness of mucosa entire area, 5 small spots into submucosa	2.0
19	” ” ” ”	”	694	surface necrosis over ¾ area of loop	1.0
20-21	” ” ” ”	”	690-692	normal mucosa	0
22	erepsin† in N/10 $NaHCO_3$	”	640	surface necrosis	1.0
23-24	” ” ” ”	”	636-638	normal mucosa	0
25	” ” ” ”	”	634	necrosis entire mucosa, some patches into muscle	3.0
26-30	” ” ” ”	”	284-632	normal mucosa	0
31-41	steapsin† in N/10 $NaHCO_3$	”	458-810	” ”	0
42-53	Amylopsin† in N/10 $NaHCO_3$	”	290-745	” ”	0
54-65	N/10 HCl	1.0	91-320	perforated ulcer in all dogs	4.0 in all dogs

* Armour, Rennet, N.F.
† Pfanstiehl.

alkaline solution alone.

Summary. 1. The proteolytic enzymes, rennin, trypsin, and erepsin, under hydrostatic pressure of 90 cm H_2O produced necrosis of the intestines of dogs, rennin being the most active of the three. 2. The average time required to produce a perforation with N/10 HCl alone was about 50% more than with rennin in N/10 HCl. 3. Neither steapsin nor amylopsin caused necrosis under the above condition.

15058

Effect of Infused Penicillin in the Bovine Mammary Gland.*

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Reports[1-3] have been published dealing with the therapy of bovine mastitis by infusion of penicillin but none has been found that deals with the physiologic behavior of this antibiotic when introduced into the mammary gland. This report deals primarily with studies on the rate of decline and irritating effects of infused penicillin. A knowledge of both of these aspects is essential to a rational approach to the use of the drug in therapy.

For the majority of experiments the sodium salt of penicillin was made up with sterile distilled water so that 1 ml of solution contained 5,000 Oxford Units. Immediately after milking infusions were made via the teat canal, in 5, 10, 30, 40, 50, 100, and 200 thousand unit doses in normal glands and in 10, 30, 50, and 100 thousand unit doses in pathologic glands. All glands were carefully palpated before and after treatment. Milk samples were taken before and for varying periods after treatment and examined grossly and for bacterial, chloride and cell content. Yields of milk before and after treatment were also ascertained. Blood and urine samples were taken periodically for penicillin determination.

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* Paper No. 2240, scientific journal series, Minnesota Agricultural Experiment Station. The work reported in this paper was made possible by a special legislative grant for the study of mastitis.

[1] Kakavas, J. C., *North Am. Vet.*, 1944, **25**, 408.

[2] Klein, L. A., Crissman, D. W., and Moor, J. W., *Am. J. Vet. Research*, 1945, **6**, 3.

[3] Bryan, C. S., Horwood, R. E., and Huffman, C. F., *Vet. Med.*, 1945, **40**, 87.

Sampling of milk for penicillin determination varied. In some trials small samples were drawn at hourly intervals, in others the intervals were longer. Complete milking varied from 10 to 72 hours after the infusions were made.

In one series the penicillin was infused in quantities of water equal to the volume of milk withdrawn.

The method used for assaying the amount of penicillin in milk was a slight modification of the cylinder-plate procedures described by Schmidt and Moyer[4] and Beadle, Mitchell, and Bonner.[5] It was noted early in the work that the zones of inhibition due to penicillin in milk stimulated the growth of the test organism (*Staphylococcus aureus* No. 209) at the periphery of the zone. In order to standardize the appearance of the zones of inhibition of the unknown and the standard solutions the following procedure was adopted. Sterile skim milk was used instead of 1% phosphate buffer for diluting the 50 Oxford Units per ml penicillin standard to 0.25, 0.5, 1.0, 2.0 and 3.0 Oxford Units per ml. These skim milk standards were run, in duplicate, on each day of assay for the standard curve[4] and all dilutions of milk samples for assay were made in sterile skim milk.

Culture dishes[5] were covered with a piece of celotex instead of a glass plate during the incubation period in an incubator humidified so that the relative humidity was between 40 and 50%.

Penicillin assay of urine samples was carried out in the same manner as for milk except 1% phosphate buffer was the diluent instead of skim milk.

Several methods were utilized at various times to assay the penicillin content in blood. The method of choice was the serial dilution assay employing *Bacillus subtilis* described by Hickey.[6] This method, in our experience, usually detects 0.031 Oxford Units per ml in blood plasma.

To determine the length of time a therapeutic level of penicillin would remain in the milk after its infusion into normal glands, small samples of milk were taken at varying intervals for penicillin determination. All of the milk was removed at intervals from 10 to 72 hours following infusion and penicillin therein determined. Twelve normal quarters were infused with solutions containing 5,000 Oxford Units per ml water and 7 normal quarters with solutions containing 100 and 200 Oxford Units per ml.

The rate of decline in concentration of penicillin per ml of milk for infusions of 5,000 and 40,000 Oxford Units in 1 and 8 ml of water respectively, is presented in Fig. 1. The rate of decrease is the greatest during the first 10 hours after which the disappearance of penicillin is at a slower rate. The rate of decrease is greater for each increment of the dose administered over the range studied which was up to 200,000 Oxford Units per quarter. In all cases where 30,000 Oxford Units or more were infused the milk contained 0.5 or more units of penicillin per ml 24 hours after the infusion was made. At no time was there sufficient penicillin in the blood to be detected by the assay methods used, but that some of the drug found its way into the blood is evidenced by its presence in the urine. The urine levels are presented in Fig. 1 for a period of 24 hours when a total of 100,000 Oxford Units were infused in all 4 quarters. The highest level of 11 Oxford

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[4] Schmidt, W. H., and Moyer, A. J., *J. Bact.*, 1944, **47**, 199.

[5] Beadle, G. W., Mitchell, H. K., and Bonner, D., *J. Bact.*, 1945, **49**, 101.

[6] Hickey, R. J., Commercial Solvents Corporation, according to information furnished in communication March 14, 1945.

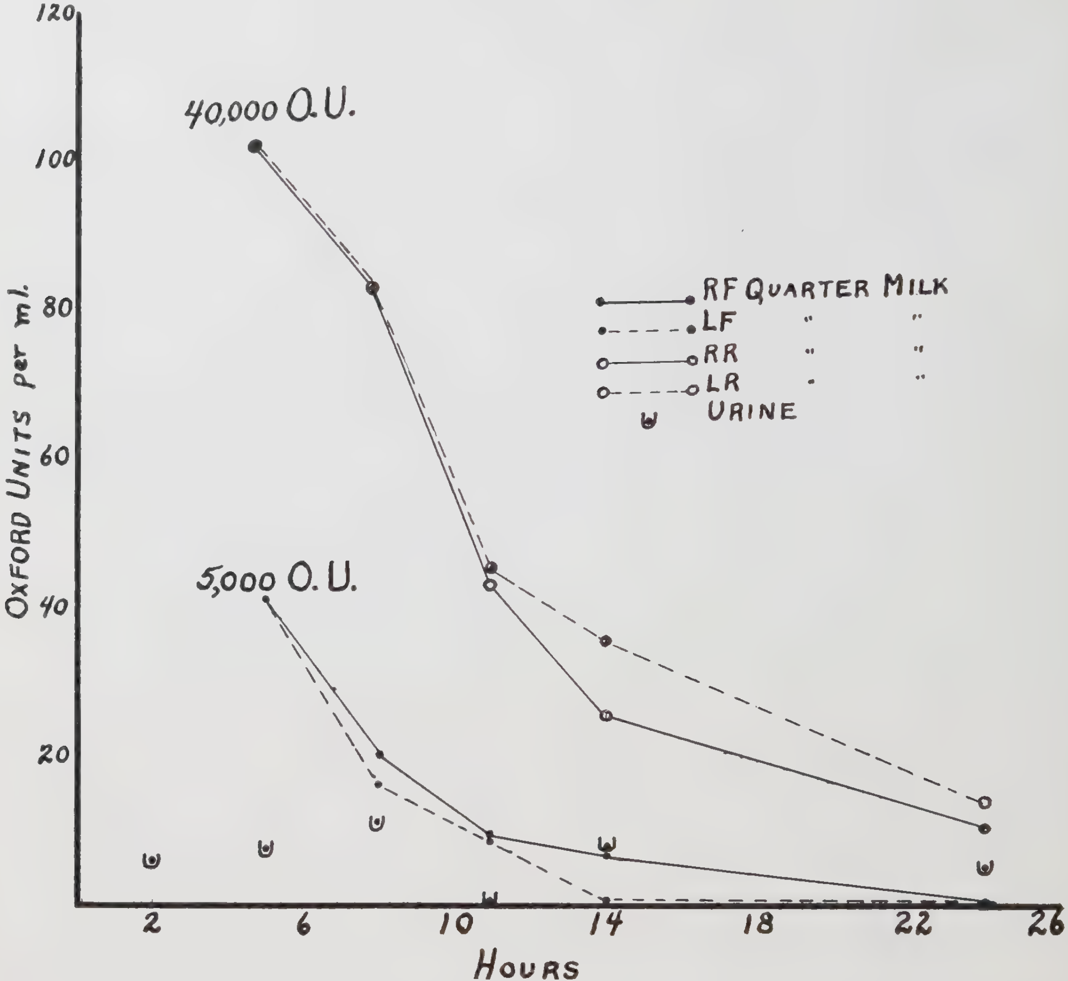

FIG. 1.
Penicillin content in milk and urine following intramammary infusions of 5,000 and 40,000 Oxford Units in 1 and 8 ml of sterile distilled water.

Units per ml urine was obtained at 8 hours and 5 Oxford Units per ml at the end of 24 hours after infusion.

The rate of disappearance of penicillin from pathologic glands presented the same picture as for the normal except in coliform infections in which the rate of decline was much greater. In the second and third infusions in consecutive 24 hour periods however, the rate of decline was progressively lower.

In order to determine whether the rate of penicillin migration out of the gland would be increased with water migration, the penicillin was infused in quantities of water equal to the quantities of milk withdrawn in 7 quarters. The results for 4 quarters are presented in Fig. 2. It will be noted that up to more than a 4-fold concentration was reached in one case 3 hours after the infusion was made. After the maximum concentration is reached the rate of decline presents much the same picture as for the experiments where the smaller amounts of water were used. The concentrations of penicillin in the urine, also presented in Fig. 2 are of the order of magnitude expected and indicate no greater rate of absorption from the gland than when small amounts of water were used.

The increased concentration of penicillin following its infusion with large quantities of water suggests that the infused water is

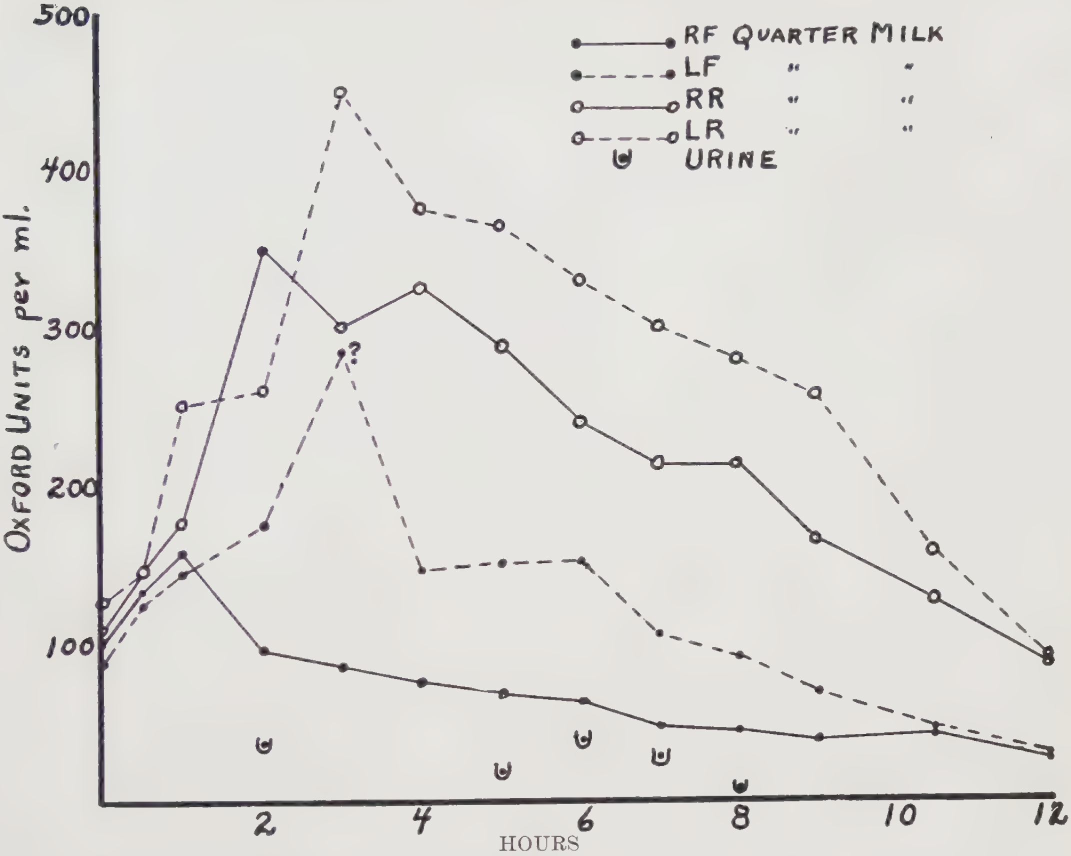

FIG. 2.

Penicillin content in milk and urine following intramammary infusions of sterile distilled water containing 100 and 200 Oxford Units per ml. The quantity of penicillin solution infused was equal to the quantity of milk withdrawn prior to the infusion.*

* RF quarter—750 ml containing 100 Oxford Units per ml. Total, 75,000 Oxford Units.
LF quarter—600 " " 100 " " " " " 60,000 " "
RR quarter—950 " " 200 " " " " " 190,000 " "
LR quarter—650 " " 200 " " " " " 130,000 " "

rapidly absorbed from the gland leaving most of the penicillin behind. Subsequent decreases in penicillin content can be accounted for chiefly by dilution with milk which is being secreted and by absorption from the gland. The absorption from the gland, however, takes place very slowly. Only when the total amount infused was 455,000 Oxford Units could any penicillin (0.06 O. U. per ml) be detected in the blood. While no evidence was obtained that penicillin is destroyed in the normal gland this possibility must be considered as another probable cause for its disappearance.

As measured by physical examination[†] of the gland, body temperature effects and changes in the character and quantity of the milk, infusion into the normal quarter of 5,000 to 200,000 units of penicillin in small quantities of distilled water had but a slight irritating effect. In some cases, no swelling could be detected. In others a slight swelling occurred in 3 to 4 hours after the infusions were made which completely subsided in 10 hours. In no case was any temperature rise noted.

In the experiments where the cows were milked at regular intervals after infusions, no demonstrable decrease in the quantity of milk was observed. In cases where the intervals between milkings were lengthened, there were decreases in the amounts of milk that could be accounted for by the intraglandular pressure resulting from the delay in withdrawing the milk.

[†] Palpation and visual aspect.

In all cases, there were observed changes in the character of the milk which varied in general with the quantity of penicillin infused. A few small flakes, with increases in chloride, and leucocytes were noted, as well as a slightly yellow color. The milk returned to normal in 3 to 4 days.

When the penicillin was infused in quantities of distilled water to equal the quantity of milk withdrawn just before the infusion was made it was followed by a significant rise in temperature with tremors and greater changes in the character of the milk. These more severe effects may be accounted for by pyrogenic properties in the water used. More experiments are in progress to clarify this point.

The majority of pathologic quarters treated were infected with coliform organisms against which penicillin is not very effective. These quarters were treated in an effort to determine what effect a very high local concentration of the drug would have upon the coliform organisms in the mammary tissue. A marked decline in temperature and an improvement in the general condition of the animal resulted following the initial infusion of penicillin in two cases of acute mastitis. However, in the majority of cases, there was no clear evidence that penicillin exerted either a favorable or an unfavorable influence upon the course of coliform mastitis. The presence of penicillin in the pathologic gland does not seem to augment the inflammatory processes already present, even though the etiologic agent is not susceptible to the drug.

Summary. Aqueous solutions of penicillin (sodium salt) containing 5,000 Oxford Units per ml had slight irritating effects on the normal bovine mammary gland as measured by physical examination of the gland, body temperature and changes in the character and quantity of milk. The largest dose infused was 40 ml (200,000 O.U.). Increase in body temperature, tremors and greater changes in the character of the milk were observed after penicillin was introduced in large volumes of water. Largest volume infused was 950 ml (190,000 O. U.). These effects may have been due to the pyrogenic properties in the water used.

In glands infused with small volumes of water containing 5,000 O. U. per ml., the rate of decline of penicillin per ml of milk is greatest during the first 10 hours after which the decline is at a slower rate. The milk contained 0.5 or more units of penicillin per ml 24 hours after the infusion of 30,000 or more units. The disappearance rate from pathologic glands was of the same order as for the normal gland except in coliform infections. When penicillin was introduced in large volumes of water the infused water was rapidly absorbed leaving most of the penicillin in the gland.

The sodium salt of penicillin was supplied through the courtesy of Drs. H. R. Stiles and Jerome Martin of the Commercial Solvents Corporation.

We are indebted to Dr. Clyde M. Christensen for counsel concerning the plate assay of penicillin. Credit is due Mrs. Ruth Ezell for technical assistance.

15059

Studies On the Inhibition of Xanthine Oxidase.*

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The formation of uric acid from xanthine and hypoxanthine depends upon the presence of xanthine oxidase. The oxidized form of *p*-aminophenol is a powerful inhibitor of xanthine oxidase activity. This has been demonstrated by Bernheim and coworkers[1] in studies with liver xanthine oxidase and by Blauch, Koch, and Hanke[2] in their work with rat blood xanthine oxidase. Since this inhibition occurs with very low concentrations of *p*-aminophenol, its effect on the uric acid production of a patient with gout was studied. Further studies, *in vitro*, of other substances which might possibly inhibit xanthine oxidase were also made.

Methods. The *p*-aminophenol was dissolved in concentrations of 0.5 to 4.0 g in 150-350 cc .1M phosphate buffer at pH 7.3 and warmed until a brown color developed. The solution was then allowed to stand exposed to the air for one hour until a brown suspension appeared. Increasing amounts of *p*-aminophenol from 0.5 g to 4.0 g were injected intravenously daily or on alternate days into a patient with very severe gout. The blood and urine uric acid was determined by the direct method of Folin.[3] The patient was maintained on a purine-free diet.

The inhibitory effect of free iodine dissolved in potassium or sodium iodide solution, on milk xanthine oxidase, was determined manometrically in the Warburg apparatus at 38°, at pH 7.3, approximating that of blood. Many other substances, including pyrogallol, were likewise tested for their inhibitory effect. The xanthine oxidase was prepared essentially by the method of Ball.[4] The enzyme was somewhat less pure than that of Ball.

Results and Discussion. At pH 7.3 a 7 x 10^{-3} M solution of *p*-aminophenol caused a 77% inhibition of milk xanthine oxidase.[4] The actual inhibition may have been considerably higher, however, since Bernheim[1] demonstrated that milk contains a substance which inactivates the oxidized form of *p*-aminophenol.

The intravenous administration of 0.5 to 4.0 g of the *p*-aminophenol did not affect the uric acid output in the urine or the blood uric acid level. During 10 days of injection the average of the daily urinary uric acid output was 405 mg, compared with corresponding values of 390 and 391 mg, for weekly periods, before and after injections, respectively. The blood uric acid remained essentially unchanged: 7.0 mg % before injection, 7.3 mg % during the injections, and a week later 6.6 mg %.

When *p*-aminophenol solutions are warmed or exposed to the air a brown color develops, indicating, according to Bernheim,[1] that the substance is partly oxidized to quinimine; the reduced and oxidized forms are thus in equilibrium, forming a reversible oxidation-reduction system.[1] Bernheim's studies indicated that the inhibition was due exclusively to the oxidized form of the drug. The negative results of intravenous injection in the human may be explained by the conversion of *p*-aminophenol to the reduced inactive form. The freshly excreted urine was clear amber in color and turned brown only upon shaking or warming, suggesting that the *p*-aminophenol

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* This study was aided by a grant from the Nutrition Research Laboratories.

The authors wish to express their appreciation to Dr. E. S. G. Barron for his valuable advice and the use of his laboratory facilities.

[1] Bernheim, F., Bernheim, M. L. C., and Michael, H., *J. Pharm. and Exp. Ther.*, 1937, **61**, 311; Bernheim, F., and Bernheim, M. L. C., *J. Biol. Chem.*, 1938, **123**, 307.

[2] Blauch, M. B., Koch, F. C., and Hanke, M. E., *J. Biol. Chem.*, 1939, **130**, 471.

[3] Folin, O., *J. Biol. Chem.*, 1933, **101**, 111; *ibid.*, 1934, **106**, 311.

[4] Ball, E. G., *J. Biol. Chem.*, 1939, **128**, 51.

TABLE I.

Effect of Iodine on Xanthine Oxidase.

0.3 cc of inhibitor; 0.1 M phosphate buffer, pH 7.3; substrate, 0.2 cc of 5 × 10-2 M xanthine; enzyme and water to 3.0 cc; 38°C; duration of experiment, 30 minutes.

Inhibitor	O_2 consumption (blank subtracted) cmm	Inhibition, %
None	122.0	
.008 M Iodine + .024 M Potassium Iodide	16.9	83.8
.006 ″ ″ + .016 ″ ″ ″	24.0	79.8
.004 ″ ″ + .012 ″ ″ ″	69.3	43.2
.002 ″ ″ + .006 ″ ″ ″	99.0	18.9
None	122.0	
.008 M Iodine + .026 M Sodium Iodide	2.3	98.2
.006 ″ ″ + .017 ″ ″ ″	5.4	95.6
.004 ″ ″ + .013 ″ ″ ″	61.3	49.8
None	58.5	
0.1 M Sodium Iodide	62.3	0
None	70.7	
0.1 M Sodium Iodide	71.9	0

was excreted in the reduced form.

The effectiveness of the drug depended also upon its diffusibility into the intact tissue cells and upon the hydrogen ion concentration. Bernheim's experiments indicated that the inhibitory effect of *p*-aminophenol was more uniform when macerated cells were used rather than tissue slices. The greatest inhibition occurred, moreover, at pH 6.4 to 6.7 and decreased considerably at the pH of the blood. These factors may be responsible for the ineffectiveness of *p*-aminophenol when injected into the human. They may also explain the relatively few toxic symptoms noted, consisting only of a transient elevation of the temperature upon 2 occasions to 100.2° and 101°, and slight nausea on one occasion.

Of a large number of substances tested manometrically for their inhibitory effect upon milk xanthine oxidase, only 2 demonstrated some inhibition. Dilutions of Lugol's solution (10% potassium iodide and 5% iodine) gave 79.8 and 83.8% inhibition with iodine concentrations of 0.006 and 0.008 M, respectively (Table I). At these concentrations of iodine in sodium iodide solution somewhat greater inhibitions (95.6 to 98.2%) were obtained, but in .002 and .004 M solutions, the inhibition was considerably reduced. Sodium iodide alone in 0.1 M solution, produced no inhibition whatever, indicating that the free iodine was the effective inhibitor of xanthine oxidase. This effect was not specific for xanthine oxidase since same concentrations of iodine inhibited other enzyme systems like uricase.

TABLE II.

Effect of Pyrogallol on Milk Xanthine Oxidase.

0.3 cc of inhibitor; 0.1 M phosphate buffer, pH 7.3; substrate, 0.2 cc of 5 × 10-2 M xanthine; enzyme and water to 3.0 cc; 38°C; duration of experiment 30 minutes.

Inhibitor	O_2 consumption (blank subtracted) cmm	Inhibition, %
None	127.3	
0.01 M Pyrogallol	45.3	64.8
None	134.3	
0.01 ″ ″	42.9	68.0
0.001 ″ ″	74.8	44.3
None	72.2	
0.01 ″ ″	24.2	67.0
0.001 ″ ″	39.8	44.9

0.001 M pyrogallol inhibited xanthine oxidase approximately 44%, a .01 M solution 65% (Table II). These concentrations of pyrogallol had no effect on partially purified uricase, but this substance is exceedingly toxic and has no clinical value.

Many other substances were tested in concentrations of 0.001 M or higher and caused no appreciable inhibition of the milk xanthine oxidase. Among the substances tested manometrically were *p*-nitrophenol, resorcinol, catechol, alpha-naphthol, *p*-bromphenol, phloroglucinol, anthraquinone, tyrosine, tryptophane, *p*-toluidine, cysteine, glycine, liver extract, alloxan, thiouracil, quinidine, neoarsphenamine, colchicine, quinine, thyroxine, and urea.

Summary. The repeated intravenous administration of increasing doses 0.5 to 4.0 g of *p*-aminophenol to a patient with gout did not affect the uric acid output in the urine or the blood uric acid level; occasionally there was transient elevation of temperature and nausea. Free iodine, in .008 M solution, caused an 88% inhibition of milk xanthine oxidase but was not specific for this enzyme. 0.001 M pyrogallol inhibited xanthine oxidase approximately 44% and had no effect upon other enzymes like uricase. Many other substances were tested and found to cause no appreciable inhibition of xanthine oxidase.

15060 P

Acute and Subacute Toxicity of Pure Citrinin.

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Citrinin is an antibiotic produced by *Penicillium citrinum*,[1] and by *Aspergillus sp.* of the *candidus* group.[2] Robinson[3] has reported on the acute toxicity of citrinin in mice following oral and intraperitoneal administration. Timonin and Rouatt[4] have reported toxicity data obtained by Noble, who administered citrinin intraperitoneally and subcutaneously to mice, intraperitoneally to rats, and orally and subcutaneously to rabbits. In the following summary the data of these investigations have been recalculated in terms of milligrams per kilogram of body weight so as to make them directly comparable with the data presented in this report.

Because of the low solubility of citrinin in water, Robinson[3] administered it to mice as a suspension in 10% gum acacia solution. In a series of 30 mice he gave the suspended citrinin orally in doses ranging from 12.5 to 500 mg per kilo of body weight. Doses of 100 mg or less produced no fatalities, but 200 and 500 mg killed 40 and 100% of the mice, respectively, in 7 days. In another series of 30 mice he found 20% and 100% mortality resulting from intraperitoneal administration of 200 and 400 mg, respectively.

In the toxicity tests reported by Timonin and Rouatt[4] the citrinin was dissolved in sodium hydroxide (pH 8-9.5) and the solution adjusted to a neutral reaction as rapidly as possible with hydrochloric acid. A series of 20 mice were given citrinin intraperitoneally in doses ranging from 25 to 125 mg per kilo of body weight. All mice receiving 100 mg died in 6 to 18 hours, and all mice receiving 125 mg died in 5 to 6 hours. None of the mice given 50 mg died after a single administration, but repetition of this dose 24 hours later produced 40% mortality. In a series of mice receiving 100 mg subcutaneously, 80% mortality resulted in 4 to 24 hours. No deaths occurred in 5 rats given 50 mg per kilo intraperitoneally. One rabbit receiving 25.7 mg per kilo in 2 doses by mouth and a second rabbit given 30 mg (absolute dose) subcutaneously daily for 5 days showed no ill effects.

Unless adequate precautions are taken, citrinin is apt to be contaminated with oxidation products and other impurities derived from the culture medium. Even pure citrinin is contaminated readily with oxidation products when it is dissolved in alcohol or in aqueous solution on the alkaline side of neutrality. The high degree of purity of the citrinin used in obtaining the following toxic-

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[1] Hetherington, A. C., and Raistrick, H., *Philosophical Transactions*, 1931, **220**, 269.

[2] Timonin, M. I., and Rouatt, J. W., *Canadian J. Public Health*, 1944, **35**, 80.

[3] Robinson, H. J., A Thesis submitted to the Graduate Faculty of Rutgers University, New Brunswick, N.J., May, 1943.

[4] Timonin, M. I., and Rouatt, J. W., *Canadian J. Public Health*, 1944, **35**, 396.

TABLE I.
Composition and Physical Characteristics of Pure Citrinin.

Product	% carbon	% hydrogen	Melting point
Theoretical	62.37	5.64	—
Hetherington and Raistrick[1]	62.20	5.63	—
” ” ”	62.32	5.58	166-170
Prepared by sublimation	62.40	5.72	167-170
Prepared by extraction	62.20	5.66	167-170

TABLE II.
Toxicity of Citrinin for Rats, Guinea Pigs, and Rabbits.

Mode of administration	Dose, mg/kg	No. of animals	No. of dead in days		
			1	7	14
Rats					
Subcutaneous	25	8	0	0	0
	50	15	0	3	3
	60	8	0	3	3
	75	8	3	5	5
	100	9	9		
	200	5	5		
Intraperitoneal	25	8	0	0	0
	50	8	0	0	0
	60	9	1	2	2
	75	12	11	12	
	100	4	4		
Guinea pigs					
Subcutaneous	25	5	0	0	0
	50	5	0	5	
	75	4	1	3	3
	100	3	1	3	
Rabbits					
Intravenous	5	5	0	0	0
	10	5	0	0	0
	15	5	0	1	1
	20	5	0	3	3
	25	1	0	1	
	50	5	0	0	5
	100	4	4		
	150	1	1		

ity data is shown in Table I. The citrinin was produced by *Penicillium citrinum* grown on asparagus juice medium by Humfeld and Feustel.[5] Citrinin purified by sublimation* under reduced pressure and by extraction has a bright lemon color with no suggestion of a brown or orange tint. The purification by extraction will be described by us in a later publication.

An aqueous solution of citrinin for toxicity studies was prepared immediately before use by dissolving a weighed amount of citrinin in the theoretically correct volume of standardized normal sodium hydroxide. With the aid of a glass electrode the pH of the solution of the sodium salt was adjusted to neutrality. Any unused solution was acidified and the citrinin recovered.

The acute toxicity of citrinin was determined in 94 rats, 17 guinea pigs, and 31 rabbits. The citrinin was administered subcutaneously to 53 rats and intraperitoneally to 41 rats. The subcutaneous route was used in guinea pigs, and the intravenous route in rabbits. The data obtained are summarized in Table II.

The subacute toxicity of citrinin injected intravenously was studied in 6 rabbits. Daily injections, except for Sunday, were continued for 8 weeks. Three rabbits received 5 mg per kilo daily for 26 days. For the next 13 days the dosage was raised to 15 mg, and finally to 20 mg for the next 13 days. Three other rabbits were given 10 mg daily for 26 days, and 20 mg daily for the next 26 days. There were no fatalities in these experiments on subacute toxicity.

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[5] Humfeld, H., and Feustel, I. C., PROC. SOC. EXP. BIOL. AND MED., 1943, **54**, 232.

* Purification of citrinin by sublimation was carried out by Dr. F. T. Jones of the Western Regional Research Laboratory. Details of the physical properties and methods of purification of citrinin are being prepared for publication.

The symptoms observed in these toxicity studies will be discussed in a later publication dealing with the tissue changes produced by citrinin, and with the pharmacological data covering effects on blood, circulation, respiration, and smooth muscle structure.

Conclusions. The lower toxicity values reported by Robinson, as compared with those reported by Timonin and Rouatt and those reported here, may be due to a low rate of absorption of citrinin from a suspension in gum acacia solution.

Citrinin in solution is rapidly absorbed, regardless of the mode of administration, as shown by the toxicity data.

The production of tissue changes and the fact that citrinin may result in delayed deaths, up to fourteen days, would make a statement regarding an LD_{50} dose misleading.

15061

Effect of Streptomycin on Avian Malaria.

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Streptomycin, an antibiotic produced by the soil actinomycete *Streptomyces griseus,* was first described by Schatz, Bugie, and Waksman.[1] Unlike penicillin, streptomycin has been shown to have a high order of activity against certain gram negative infections both *in vitro* and *in vivo*.[2,3,4] Streptomycin has also been found to be active against the acid fast organism *Mycobacterium tuberculosis* both *in vitro*[5] and in the experimental animal.[6] In this communication we are reporting the results of studies on the activity of this antibiotic against avian malaria.

Trophozoite induced *Plasmodium gallinaceum* infections were established in 50 g Single Comb White Leghorn chicks by an intravenous inoculum of 200,000,000 parasitized erythrocytes per kilogram. The tests for suppressive activity against both trophozoite induced *P. cathemerium* and *P. lophurae* malaria were performed on 50 g Pekin ducklings inoculated intravenously with 500,000,000 parasitized erythrocytes per kilogram. The sporozoite induced *P. gallinaceum* infections used for the prophylactic tests were established in 50 g Single Comb White Leghorn chicks by the intravenous inoculation of 0.2 cc per bird of a suspension of sporozoites prepared by grinding 100 infected mosquitoes in 20 cc of chicken plasma.

The streptomycin used in these experiments was supplied by Merck & Co., Inc. and came from batch 162 which had a potency of 440 units per mg.[2] In all cases the streptomycin was administered as an aqueous solution intramuscularly every 3 hours. Birds inoculated with the trophozoites of *P. cathemerium, P. gallinaceum* and *P. lophurae* were treated for 5 days while the birds inoculated with the

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[1] Schatz, A., Bugie, E., and Waksman, S. A., Proc. Soc. Exp. Biol. and Med., 1944, **55**, 66.

[2] Jones, D., Metzger, H. J., Schatz, A., and Waksman, S. A., *Science*, 1944, **100**, 103.

[3] Robinson, H. J., Smith, D., and Graessle, O., Proc. Soc. Exp. Biol. and Med., 1944, **57**, 226.

[4] Heilman, F. R., *Proc. Staff Meet., Mayo Clinic*, 1944, **19**, 553.

[5] Schatz, A., and Waksman, S. A., Proc. Soc. Exp. Biol. and Med., 1944, **57**, 244.

[6] Feldman, W. H., and Hinshaw, H. C., *Proc. Staff Meet., Mayo Clinic*, 1944, **19**, 593.

TABLE I.
Ineffectiveness of Streptomycin Against Trophozoite-Induced Avian Malaria Infections.

No. of birds	Drug	Dose	Route	% r.b.c parasitized at peak of infection
P. cathemerium in Pekin ducklings.				
5	Streptomycin	4 × 100,000 units/kg	i.m.	33.4
5	,,	2 ,, ,,	,,	41.2
5	,,	1 ,, ,,	,,	29.3
5	Quinine	80 mg/kg	p.o.	0.3
5	Controls	——	—	37.2
P. gallinaceum in Single Comb White Leghorn chicks.				
5	Streptomycin	4 × 100,000 units/kg	i.m.	70.7
5	,,	2 ,, ,,	,,	70.2
5	,,	1 ,, ,,	,,	68.9
5	Sulfadiazine	0.05% ,,	diet	1.1
10	Controls	——	—	66.5
P. lophuræ in Pekin ducklings.				
5	Streptomycin	4 × 100,000 units/kg	i.m.	73.6
5	,,	2 ,, ,,	,,	73.4
5	,,	1 ,, ,,	,,	74.5
5	Quinine	40 mg/kg	p.o.	0.1
5	Controls	——	—	74.6

TABLE II.
Effect of Streptomycin Against Sporozoite-Induced *P. gallinaceum* Malaria.

No. of chicks	Drug	Dose, units/kg or % in diet	Route	% r.b.c. parasitized on 9th day
5	Streptomycin	40 × 10,000	i.m.	17.7
5	,,	20 ,,	,,	26.9
5	,,	10 ,,	,,	31.8
5	,,	5 ,,	,,	38.5
5	,,	2.5 ,,	,,	40.8
	Sulfadiazine	0.2%	diet	0
	,,	0.1%	,,	0
10	Controls	—	—	35.1

sporozoites of *P. gallinaceum* were treated for 3 days. Treatment was invariably begun within an hour following the inoculation. In the experiments with each infection, groups of 5 birds were given 400,000, 200,000, 100,000, 50,000 and 25,000 units respectively per kilogram per day. In each experiment 5 infected birds served as untreated controls and 5 infected birds were given either quinine or sulfadiazine.

The effect of streptomycin therapy was judged by parasite counts made on the fifth day in the *P. cathemerium* experiment and on the eighth day *P. lophurae* experiment. Parasite counts on the trophozoite induced *P. gallinaceum* infection were made on the fifth day, while the counts on the sporozoite induced infection were made on the ninth day. The parasite counts were made by determining the number of parasitized erythrocytes among ten thousand erythrocytes on Giemsa stained thin blood smears.

Streptomycin at a dose as high as 400,000 units per kilogram per day showed no suppressive activity against *P. cathemerium*, *P. gallinaceum* or *P. lophurae* infections (Table I). The partially purified streptomycin used in these experiments did, however, have a slight effect on the sporozoite induced *P. gallinaceum* infection (Table II). While the group of birds on each dose level was so small that the differences among the average parasite counts are not in themselves significant, the linear relationship between the dose of the drug and the parasite count is noteworthy.

15062

A Further Study of the Lability of the Methyl Group of Creatine.

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It has been shown by du Vigneaud, Chandler, and Moyer[1] that neither creatine nor creatinine will support the growth of young rats maintained on an otherwise methyl-free diet in which homocystine is the only sulfur-amino acid. The inability of rats to grow under these conditions was indicative of the non-reversibility of transmethylation from methionine[2,3] and choline[3] to creatine. However, it was recognized[1] that the transfer of methyl groups from creatine to choline and methionine might occur, but not at a rate fast enough to support growth. To test this possibility, creatine containing the deuteriomethyl group was synthesized and fed to young rats to see whether it could act as a methyl donor for choline synthesis.

Synthesis of deuteriocreatine. Deuteriosarcosine hydrochloride was synthesized by the method of Fischer and Bergmann[4] using deuteriomethyl iodide[3] for the methylation of toluenesulfonyl glycine. Deuteriosarcosine then was prepared from the hydrochloride by treatment with Ag_2O, and was converted to deuteriocreatine by treatment with cyanamide in the presence of a trace of ammonia.[5] The deuteriocreatine contained 81.0 atom % deuterium in the methyl group.

Analysis. Deuteriocreatine. Calculated, N 31.46; found, N 31.53.

Feeding experiment. Two albino rats weighing about 50 g were placed in individual cages arranged for urine collection and were fed a basal diet containing homocystine but lacking methionine, choline, and betaine. The basal diet was the same as that employed in the previous growth studies with ordinary creatine.[1] Deuteriocreatine was mixed in the diet at a level of 1.75 parts per hundred. This provided a daily supplement of creatine equivalent in methyl groups to approximately 25 mg of choline chloride. For the feeding experiment, the synthetic deuteriocreatine was diluted with a sufficient amount of ordinary creatine in order to lower the deuterium content of the methyl group from 81.0 to 64.3 atom %.

The changes in body weight and amounts of test compound ingested during the feeding period are given in Table I. The rats were sacrificed at the end of 7 days and choline and creatine were isolated from the tissues of each by the procedures described in a previous investigation.[3] Choline was isolated as choline chloroplatinate, and creatine as creatinine potassium picrate. The purity of the latter compound was determined colorimetrically by the Jaffe reaction.

Analysis. Choline chloroplatinate.

Rat No. 1012 Calculated, Pt. 31.66; found, Pt. 31.41.

Rat No. 1013 Calculated, Pt. 31.66; found, Pt. 31.63.

The urine excreted by both rats over the entire experimental period was collected and pooled, and creatinine was isolated from it as creatinine potassium picrate.[6] The deuterium content of each of the isolated compounds is given in Table I.

Discussion. As the data in Table I show, the tissue choline contained no deuterium. The methyl group of creatine, therefore, is not available for the synthesis of choline, and transmethylation from choline to creatine is

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* The authors wish to express their appreciation to the Nutrition Foundation, Incorporated, for a research grant that has aided greatly in this work.

[1] du Vigneaud, V., Chandler, J. P., and Moyer, A. W., *J. Biol. Chem.*, 1941, **139**, 917.

[2] Borsook, H., and Dubnoff, J. W., *J. Biol. Chem.*, 1940, **132**, 559.

[3] du Vigneaud, V., Cohn, M., Chandler, J. P., Schenck, J. R., and Simmonds, S., *J. Biol. Chem.*, 1941, **140**, 625.

[4] Fischer, E., and Bergmann, M., *Ann. Chem.*, 1913, **398**, 96.

[5] Rosengarten, F., and Strecher, A., *Ann. Chem.*, 1871, **157**, 1.

TABLE I.

Feeding of Deuteriocreatine.

Deuteriocreatine containing 64.3 atom % deuterium in the methyl group was fed for 7 days.

			Deuterium content of isolated compounds		
Rat No.	Change in body wt, g	Total deuteriocreatine ingested, mg	Choline chloroplatinate from tissues, atom %	Creatinine K picrate from tissues, atom %	Creatinine K picrate from urine, atom %
1012	55-58	542	0	7.3 ±.1	11.5 ±.2
1013	50-54	438	0	6.7 ±.1	

thus irreversible.

The creatinine potassium picrate isolated from the rat tissues at the end of the experimental period contained about 7 atom % deuterium. The creatinine potassium picrate isolated from the pooled urine of the entire experimental period contained even more deuterium, *i.e.*, 11.5 atom %. These data on urinary creatinine agree with the findings of Bloch and Schoenheimer.[6] These investigators found that although urinary creatinine from rats on a *creatine-free* diet is derived solely from tissue creatine, the urinary creatinine excreted during the feeding of relatively large amounts of creatine labeled with N^{15} contained more than the expected amount of N^{15}. Bloch and Schoenheimer, therefore, suggested that a small portion of dietary creatine is converted to creatinine and excreted before the dietary creatine is completely admixed with the creatine in the tissues. Our results offer support for this suggestion; for, in the absence of the direct conversion and excretion of a fraction of the deuteriocreatine of the diet, the deuterium content of the urinary creatinine should have been 3.5 to 4 atom %, rather than the 11.5 atom % actually found.

It is of interest to point out that on the basis of the amount of deuterium in the tissue creatine, an average of 43% of the tissue creatine was derived from the deuteriocreatine of the diet in 7 days.

Summary. Creatine containing the deuteriomethyl group was fed to two young rats on an otherwise methyl-free diet. The creatine isolated from the tissues contained a considerable amount of deuterium, while the choline from the tissues was completely devoid of deuterium. Transmethylation from choline to creatine, therefore, is irreversible.

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[6] Bloch, K., and Schoenheimer, R., *J. Biol. Chem.*, 1939, **131**, 111.

15063

Inhibition of Pigment Deposition in Incisor Teeth of Rats Deficient in Vitamin E from Birth.*

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Feeding fat-containing vitamin E-deficient diets to rats from weaning results in disappearance of the normal yellow-brown pigment of maxillary incisor teeth in about 20 days.[1] The present study was designed to determine the effect of similar diets on incisor pigmentation from birth.

For this purpose, 14 female albino rats were mated with normal males and maintained on a normal stock diet until delivery; then, with their offspring, they were divided into 6 groups and maintained for a 60-day period on the following rations: group I (3 females with 26 young): sucrose 65%; casein, alcohol-extracted, 20%; dried yeast, ether-extracted, 10%; salt mixture no. 2, U.S.P., 5%; tetrasodium salt of 2-methyl-1,4-naphthohydroquinone diphosphoric acid, 1 mg %; vitamin A and D concentrate in oleic acid, 2 drops weekly per animal corresponding to 560 i.u. A and more than 80 i.u. D. Group II (3 females with 29 young): same as group I but with 20% sucrose replaced by 20% cod liver oil. Group III (3 females with 29 young): same as group I but with 20% sucrose replaced by 20% lard. Group IV (2 females with 26 young): same as group I plus 10 mg % of *d,l*-alpha-tocopherol acetate. Group V (2 females with 31 young): same as group II plus 10 mg % of *d,l*-alpha-tocopherol acetate. Group VI (2 females with 29 young): same as group III plus 10 mg % of *d,l*-alpha-tocopherol acetate. The young received milk from the mothers, but after the first two weeks gradually began to eat of the diets themselves.

From about the 24th day, the offspring of the mothers from groups I (vitamin E-deficient, fat-free), IV (vitamin E-sufficient, fat-free), V (vitamin E-sufficient, 20% cod liver oil), and VI (vitamin E-sufficient, 20% lard) developed incisor enamel pigment at the normal rate (Fig. 1) as measured by a method previously described.[1] No pigment developed in the incisor teeth of either jaw in the young of groups II (vitamin E-deficient, 20% cod liver oil) and III (vitamin E-deficient, 20%

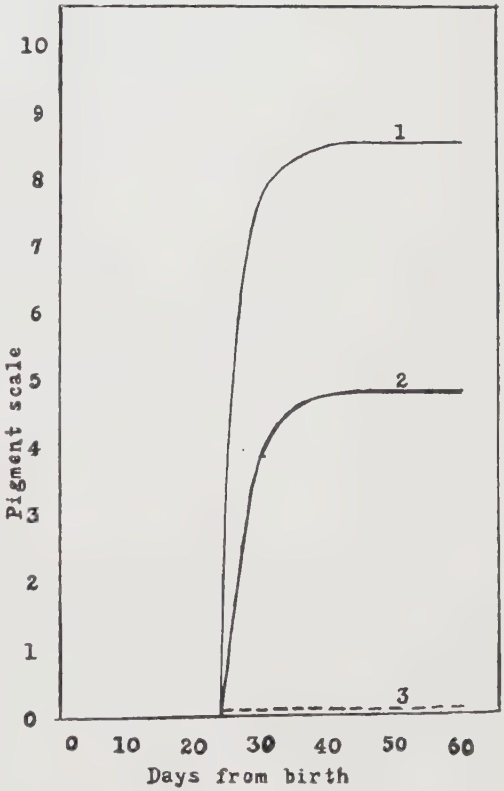

FIG. 1.

Lines 1 (maxillary) and 2 (mandibular) represent degree of pigmentation in the incisors of the offspring of animals 1, 4, 5, and 6. Line 3 indicates incisor pigmentation in the young of 2 and 3.

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* Aided by grants of the Carnegie Corporation of New York, the Eastman Dental Dispensary of Rochester, and Wyeth Incorporated of Philadelphia.

[1] Granados, H., and Dam, H., *Science*, 1945, **101**, 250.

lard). However, incisor pigmentation in the mothers of these last 2 groups was not influenced during the experimental period.

That the incisor teeth of the young of group I, receiving the E-deficient fat-free ration, were not depigmented demonstrates a direct causal relationship between fat in the diets of mothers II and III, and failure of enamel pigmentation in their offspring. Further, the results indicate that the types of fat represented by cod liver oil and lard function essentially alike in inhibiting incisor pigmentation in E-deficient rats. Necessity for *dietary* fat in the prevention of pigment deposition when vitamin E-deficient rations are given from birth is disclosed by the fact that consumption of fat contained in the milk of mothers of group I, fed a fat-free diet, did not inhibit the development of pigment in the offspring.

Maintenance of normal incisor pigmentation in mothers II and III during the experimental period shows that the age at which the vitamin E-free, high fat diets are given, influences the disappearance of the pigment. Depigmentation occurs in rats reared on similar diets for 159 days[2] from weaning, but is limited to maxillary incisors, indicating that the enamel organ of the mandibular incisors has a prolonged ability to continue the formation and deposition of pigment. Apparently, the absence of deposition in both maxillary and mandibular incisors also depends upon the age at which the experimental diets are given.

Evidence has been reported[3,4,5] that iron is an important component of the enamel pigment; that the color intensity is directly proportional to the iron content of the enamel; and that unpigmented enamel encountered under certain circumstances has a decreased iron content. It seems likely, therefore, that dietary fat in E-deficient rats affects the normal ability of ameloblasts to produce or deposit the iron-containing pigment.

The brown pigment of adipose tissue[6] developed as early as the 21st day of age in offspring of group II, fed a high cod liver oil, vitamin E-deficient diet; but not in the young of III, on the E-deficient lard ration. A difference in the effects of the two fats on the production of adipose pigment is apparent, whereas their influences are alike in regard to incisor pigment.

Summary. Fourteen normal female rats, in the presence of their offspring, were maintained for 60 days from delivery on vitamin E-deficient rations containing no fat, 20% cod liver oil, and 20% lard respectively. Production of normal pigment failed to occur in the enamel of the incisor teeth of the young of animals receiving cod liver oil and lard.

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[3] Ratner, S., *J. Den. Res.*, 1935, **15**, 89.

[4] Murray, M. M., and Glock, G. E., *Brit. Den. J.*, 1939, **66**, 345.

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[6] Dam, H., *J. Nutrition*, 1944, **27**, 193.

15064

Failure of Succinylsulfathiazole to Aggravate Egg White Induced Biotin Deficiency in Rats.

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Manifestations of deficiency disease may be induced in the rat by the feeding of sulfonamide-containing purified rations.[1,2] Daft and co-workers[3] demonstrated that the syndrome was ascribable, in part, to a lack of biotin. Nielsen and Elvehjem[4] confirmed this finding and, in addition, reported that a "folic acid" concentrate had an even more marked effect in reversing the action of the sulfa drug than did biotin. Martin[5] and Welch and Wright[6] likewise observed the counteracting effect of these vitamins upon the action of the sulfonamide.

Biotin deficiency in the rat can also be induced by the feeding of an egg white containing purified diet. The depletion signs thus produced may be prevented or overcome by biotin thus demonstrating the existence of a single deficiency. Untreated animals decline in weight, show a progressive alopecia and dermatitis and death finally ensues. On the other hand, the sulfonamide induced syndrome is complicated by a co-existing "folic acid" deficiency. Only the initial signs of biotin depletion (mole-like pelage and spectacled eye) are apparent; general alopecia and exudative dermatitis are not seen. The animals succumb from the dyscrasia[7,8] resulting from a lack of "folic acid".[9]

The experiments herein described were designed to demonstrate the influence of succinyl-sulfathiazole upon the progress of biotin depletion in rats receiving egg white containing diets with and without a source of "folic acid."

Experimental and Results. Two diets, employed routinely in the production of biotin deficiency, have been used in these studies. The rations were identical except that one contained 2% of dried beef liver replacing an equivalent amount of sucrose. This dried liver contained 2.4 μg of biotin and 31 μg of "folic acid" per gram.* Succinylsulfathiazole was incorporated in the rations as indicated at a level of 0.75%.

The composition of the diets is as follows:

	23	23S	24	24S
Dried egg white	15	15	15	15
Casein (Labco)	15	15	15	15
Sucrose	52	51.25	54	53.25
Salts (U.S.P. No. 1)	4	4	4	4
Hydrogenated veg. oil	10	10	10	10
Cod liver oil	2	2	2	2
Dried beef liver	2	2	—	—
Succinylsulfathiazole	—	0.75	—	0.75

All of the rations were supplemented with 0.8 mg each of thiamine and pyridoxine, 1.6 mg of riboflavin, 5 mg of Ca pantothenate, 10 mg of nicotinamide and 100 mg of choline chloride per 100 g.

Male rats, placed at weaning on the several diets, were observed for a period of 42 days. As can be seen from Table I the feeding of the sulfonamide in conjunction with the egg white liver containing ration (23S) did not shorten the depletion period required for the production of the syndrome ascribable to a

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[1] Black, S., McKibbin, J. M., and Elvehjem, C. A., PROC. SOC. EXP. BIOL. AND MED., 1941, **47**, 308.

[2] Welch, A. D., *Fed. Proc.*, 1942, **1**, 171.

[3] Daft, F. S., Ashburn, L. L., and Sebrell, W. H., *Science*, 1942, **96**, 321.

[4] Nielsen, E., and Elvehjem, C. A., *J. Biol. Chem.*, 1942, **145**, 713.

[5] Martin, G. J., PROC. SOC. EXP. BIOL. AND MED., 1942, **51**, 353.

[6] Welch, A. D., and Wright, L. D., *J. Nutr.*, 1943, **25**, 555.

[7] Spicer, S. S., Daft, F. S., Sebrell, W. H., and Ashburn, L. L., *Public Health Rep.*, 1942, **57**, 1559.

[8] Kornberg, A., Daft, F. S., and Sebrell, W. H., *Science*, 1943, **98**, 20.

[9] Daft, F. S., and Sebrell, W. H., *Public Health Rep.*, 1943, **58**, 1542.

* The "folic acid" determination was carried out with *Lactobacillus casei* and unitage was based on a "potency of 40,000."[10]

TABLE I.
Biotin Deficiency in Rats Fed Egg White-Succinylsulfathiazole Rations.

Diets (Groups of 5 male rats each)	Gain in wt, 42 days, g	No. survivors	Biotin deficiency first observed days
Diet 23	91	3	24
Diet 23 + 5 μg biotin daily	238	5	—
Diet 23S	114	5	26
Diet 23S + 5 μg biotin daily	229	5	—
Diet 24	87	5	24
Diet 24 + 5 μg biotin daily	203	5	—
Diet 24S	72	4	24
Diet 24S + 5 μg biotin daily	132	5	—
Diet 24S + 25 μg "folic acid"	103	5	28
Diet 24S + 5 μg biotin + 25 μg "folic acid"	206	5	—

deficiency of biotin: furthermore the average weight increment was augmented slightly by the feeding of the drug. This "growth stimulating" effect of succinylsulfathiazole has been observed routinely in this laboratory with rats receiving purified diets containing liver. Alopecia and depression of growth were prevented by the prophylactic oral administration of 5 micrograms of biotin daily. Thus, it appears that succinylsulfathiazole, at the level fed, produced no untoward effects in rats receiving the egg white liver containing ration providing that biotin was supplied.

The sulfonamide had a profound inhibiting effect upon growth when fed in conjunction with the egg white containing ration in which the dried whole liver was replaced by sucrose (24S). The depletion period, however, was not shortened nor were the lesions more marked. The daily feeding of 5 micrograms of biotin prevented the appearance of the dermatitis ascribable to a lack of the vitamin but did not evoke a growth response comparable with that observed in the absence of the sulfonamide. The feeding of a "folic acid" concentrate, containing 0.20 μg of biotin and 25 μg of "folic acid"[10] by microbiological assay, likewise produced only an incomplete growth response. However, when 5 μg of biotin was fed daily in conjunction with this concentrate the growth response equalled that observed in the absence of the sulfonamide. The rate of growth was somewhat less than that noted with the dried liver containing diet (23) supplemented with biotin. However, the intakes of both "folic acid" and biotin were in excess of the amounts of these vitamins supplied in the 2% liver present in diet 23.

Summary. The feeding of succinylsulfathiazole in conjunction with an egg white containing diet with or without liver, did not aggravate the signs of biotin depletion in the rat nor was the period required to produce such deficiency manifestations shortened.

When biotin was administered in conjunction with the sulfonamide ration with liver, normal growth resulted. Biotin and a "folic acid" concentrate, when fed separately as adjuncts to the diet without liver, gave only minimal growth responses. The feeding of both vitamins produced a growth response that almost equalled that observed with rats receiving the liver containing ration supplemented with biotin.

We wish to thank Dr. J. L. Stokes of the Microbiological Laboratories of Merck & Co., Inc., for the microbiological assays and Dr. J. C. Keresztesy of the Research Laboratories of Merck & Co., Inc., for the "folic acid" concentrate.

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[10] Mitchell, H. K., and Snell, E. E., *Univ. of Texas Publication*, 1941, **4137**, 36.

15065

Non-Availability of Fecal Thiamine in Thiamine Deficiency.

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That fecal thiamine and cocarboxylase are not available to man was suggested recently by Alexander and Landwehr.[1] These investigators believed that the thiamine so excreted was present largely in the bodies of bacteria and other organisms. Furthermore, an amount of thiamine and cocarboxylase approximating that normally excreted was not absorbed when administered in a retention enema. On the other hand, Najjar and Holt[2] concluded that the large intestine could absorb thiamine as evidenced by the increased urinary excretion of thiamine following the administration of a large quantity of the vitamin by rectum.

Williamson and Parsons[3] have reported that the ingestion of pineapple fiber resulted in an increased fecal elimination of thiamine in human subjects. Increasing the thiamine intake alone did not affect an increased fecal excretion.

Leong[4] found that the fecal excretion of thiamine by rats was negligible if the intake was less than 30 International units (90 μg) daily. Incomplete absorption occurred if this level were exceeded. Guerrant and co-workers[5] have shown that the type of carbohydrate in the ration has a marked influence on the synthesis of thiamine in the digestive tract of the rat. The effect of coprophagy was less marked when the diet contained sucrose, glucose or commercial cornstarch than when dextrinized cornstarch was employed.

Experiments conducted in this laboratory indicate that thiamine-depleted rats, in which commercial cornstarch served as the carbohydrate, excrete appreciable quantities of this vitamin in their stools; that the total thiamine as measured following enzymatic hydrolysis of the feces is not nutritionally available is evidenced by the progress of the depletion state in the animals terminating in polyneuritis and death.

Weanling male rats were segregated into 3 like groups of 8 animals each and were placed on a thiamine low diet of the following composition: casein (technical) 16; cornstarch 60; autoclaved brewers' yeast, 9; salt mix U.S.P. No. 1, 4; crisco, 9; cod liver oil, 2; and non-irradiated brewers' yeast, 0.2.

One group of rats received no supplementation and served as controls while the 2 thiamine-dosed groups were given a sub-optimal level (5 μg) or an excess of the vitamin (50 μg) daily by stomach tube.

The fecal thiamine excretion was followed throughout the course of the experiment; the thiochrome method of Hennessy[6] being employed in the determination.

The rats receiving the thiamine-deficient diet excreted essentially the same total thiamine per g of feces throughout the experimental period as did the animals receiving 50 μg of thiamine (Table I). The quantity of feces decreased in the thiamine low group as the depletion progressed. The fecal output for the several groups followed the weight changes. The larger animals (those receiving thiamine) excreted more feces than did the depleting rats. The food consumption governed the fecal output.

Cocarboxylase was determined on the thirty-first day of the test. At this time the animals receiving the deficient regimen had

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[1] Alexander, B., and Landwehr, G., *Science*, 1945, **101**, 230.

[2] Najjar, V. A., and Holt, L. E., Jr., *J. Am. Med. Assn.*, 1943, **123**, 683.

[3] Williamson, A., and Parsons, H. T., *J. Nutr.*, 1945, **29**, 51.

[4] Leong, P. C., *Biochem. J.*, 1937, **31**, 373.

[5] Guerrant, N. B., Dutcher, R. A., and Tomey, L. F., *J. Biol. Chem.*, 1935, **110**, 233.

[6] Hennessy, D. J., *Cereal Chem. Bull.*, 1942, **2**, 25.

TABLE I.
Fecal Thiamine Excretion of Rats with Varying Thiamine Intakes.

Days on test	Group	No. of rats	Avg wt, g	Avg 24 hr fecal output, g	Total thiamine, µg/g feces	Cocarboxylase % of total thiamine
1st day	Control period	8	46	0.28	12.9	—
10th	Thiamine low	8	79	0.19	22.2	—
	5 µg thiamine	8	68	0.47	7.2	—
	50 ” ”	8	82	0.54	24.0	—
23rd	Thiamine low	8	81	0.21	12.9	—
	5 µg thiamine	8	115	0.51	11.3	—
	50 ” ”	8	136	0.65	21.3	—
31st	Thiamine low	7	67	0.11	15.1	80
	5 µg thiamine	7	132	0.51	12.6	69
	50 ” ”	7	176	0.83	16.6	41
42nd	Thiamine low	2	77	0.10	17.5	—

declined in weight and one of the group had succumbed to thiamine deficiency. These rats excreted 80% of their total thiamine in the form of cocarboxylase as contrasted with 69% for the animals receiving 5 µg of thiamine and 41% for the rats receiving 50 µg of thiamine daily.

After 42 days on test 6 of the animals in the original group had succumbed. The 2 remaining rats although evidencing signs of severe thiamine deficiency still excreted an appreciable quantity of total thiamine in their stools.

The non-availability of fecal thiamine was further demonstrated by the feeding of feces to thiamine-depleted animals. Five rats, placed at weaning on the thiamine low diet, were maintained on the regimen until a plateau in weight had been reached (25 days). Each animal was then dosed by stomach tube with a suspension of feces from a like group of thiamine-deficient rats. The feces were fed in a quantity sufficient to supply 5 µg of thiamine daily (as determined by the thiochrome method). The feeding of the thiamine containing feces was without effect upon the progress of the deficiency. However, when 5 µg of thiamine was administered daily spectacular weight gains were observed.

Summary. Rats maintained on a thiamine-deficient diet excreted essentially the same concentration of thiamine in their stools as did animals receiving 5 or 50 µg of thiamine daily. The output of feces was greatly reduced in the thiamine low group. During the later stages of the depletion the thiamine was present largely in the form of cocarboxylase.

The fecal thiamine of the deficient rats was not available when administered curatively by the oral route to animals which had plateaued in weight on a thiamine-low diet.

We are indebted to Miss Frances Lewis and Mr. Ling Chen for their technical assistance.

15066

Fecal Riboflavin of Rats Receiving Varying Intakes of Riboflavin.

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The biosynthesis of vitamin B factors was suggested by Roscoe[1] who observed that coprophagous rats maintained on diets containing cornstarch lived and in some cases grew despite the absence of the vitamin B complex from their rations. She postulated that the B vitamins were probably synthesized in the lumen of the gut. Guerrant and Dutcher,[2] in experiments in which vitamin B (thiamine) was supplied as an alcoholic extract of yeast, found that the feces from vitamin G (riboflavin) deficient rats supplied this factor. The stools of depleting rats did not decrease in riboflavin content as the deficiency progressed. The same investigators[3] later observed that the synthesis of riboflavin was influenced by the source of carbohydrate in the diet. Dextrinized cornstarch favored riboflavin production while sucrose did not. Mannering and co-workers[4] reported that, in general, those carbohydrates which decreased the amount of dietary riboflavin needed by the rat for growth were also responsible for the greatest quantities of riboflavin in the feces. Najjar *et al.*[5] in studies with humans reported the maintenance of a normal fecal excretion of riboflavin although the purified diet fed contained only small amounts of this vitamin.

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[1] Roscoe, M. H., *Biochem. J.*, 1931, **25**, 2056.

The above experiments, although affording adequate evidence for the synthesis of riboflavin do not constitute proof for the absorption of the vitamin from the large intestine. Selye,[6] working with bilaterally nephrectomized rats, obtained results indicating the destruction of riboflavin in the cecum; however, physiological amounts of riboflavin could not have been detected with the techniques employed.

In the present experiments the fecal excretion of riboflavin has been followed in rats receiving varying levels of this vitamin. These experiments were designed to show the effect of riboflavin intake upon fecal riboflavin excretion.

Experimental. Male rats were placed at weaning on Diet 30, the composition of which is as follows: casein (Labco) 18%; dextrose, 68%; salt mix, U.S.P. No. 1, 4%; crisco, 8%; cod liver oil, 2%; and supplemented with 0.8 mg each of thiamine and pyridoxine; 10 mg nicotinamide, 5 mg calcium pantothenate and 100 mg of choline chloride per 100 g of diet.

Animals receiving riboflavin were dosed daily by stomach tube with 10 μg or 40 μg of this factor. The rats receiving riboflavin were segregated into 2 groups; the one was fed the basal ration *ad libitum* and with the other the caloric intake was restricted to 50% of that consumed by the corresponding *ad libitum* group. In a previous experiment an attempt was made to maintain riboflavin fed animals on the same caloric intake as the deficient group. All rats thus restricted succumbed after 3 to 7 days on test. It should be mentioned in this connection that the progress of the deficiency state attributable to riboflavin-avitaminosis is unique in that weight gains are minimal and the food consumption is correspondingly low.

Fecal riboflavin was determined by the fluorometric method at intervals as indicated in Table I. The concentration of riboflavin as expressed in micrograms per gram of feces was essentially the same for all groups and was therefore independent of the riboflavin intake. Furthermore, the riboflavin content of the feces remained constant in the depleting group (A), despite the development of the deficiency state. The rats, in both groups (C and E) in which the caloric intake had been restricted, excreted less total riboflavin than did the animals (B and D) receiving the same intake of the vitamin but with the basal ration supplied *ad libitum.* In those animals receiving riboflavin with food supplied *ad libitum* the total daily fecal excretion of riboflavin exceeded that of the deficient and restricted groups. Thus the output of riboflavin in the stools more nearly followed the food consumption than the riboflavin intake.

It is of interest to note that Silber[7] observed that the fecal pantothenic acid excretion of dogs maintained on a pantothenic acid

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[2] Guerrant, N. B., and Dutcher, R. A., *J. Biol. Chem.*, 1932, **98**, 225.

[3] Guerrant, N. B., and Dutcher, R. A., PROC. SOC. EXP. BIOL. AND MED., 1934, **31**, 796.

[4] Mannering, G. J., Orsini, D., and Elvehjem, C. A., *J. Nutr.*, 1944, **28**, 141.

[5] Najjar, V. A., Johns, G. A., Medairy, G. C., Fleischmann, G., and Holt, L. E., Jr., *J. Am. Med. Assn.*, 1944, **126**, 357.

[6] Selye, H., *J. Nutr.*, 1943, **25**, 137.

[7] Silber, R. H., *J. Nutr.*, 1944, **27**, 425.

TABLE I.
Fecal Riboflavin Excretion of Rats Receiving Varying Intakes of Riboflavin.

Days on test	Group	No. of rats	Avg wt, g	Avg 24 hr fecal output, g	Riboflavin, μg/g feces	Riboflavin total output, μg/rat/day
1st day	Control period	9	42	0.15	27	4.1
12th	A	9	57	0.24	58	13.9
	B	9	81	0.25	23	5.8
	C	9	68	0.21	34	7.1
	D	7	92	0.43	—	—
	E	9	72	0.15	46	6.9
19th	A	8	55	0.18	21	3.8
	B	9	91	0.37	25	9.3
	C	9	76	0.22	20	4.4
	D	7	117	0.43	24	10.3
	E	8	82	0.14	26	3.6
26th	A	7	59	0.22	28	6.2
	B	9	117	0.46	26	12.0
	C	9	91	0.32	28	9.0
	D	7	144	0.50	25	12.5
	E	8	99	0.29	29	8.4
33rd	A	7	58	0.14	31	4.3
	B	9	126	0.35	38	13.3
	C	9	100	0.22	31	6.8
	D	7	164	0.56	25	14.0
	E	8	111	0.20	28	5.6
40th	A	6	59	0.16	27	4.3
	B	9	136	0.32	23	7.4
	C	9	106	0.20	23	4.6
	D	7	183	0.50	40	20.0
	E	8	119	0.20	29	5.8

Key to groups:
A—Riboflavin deficient.
B—10 γ riboflavin daily.
C—10 γ riboflavin daily, caloric intake restricted to 50% *ad lib.*
D—40 γ riboflavin daily.
E—40 γ riboflavin daily, caloric intake restricted to 50% *ad lib.*

deficient diet was the same when expressed in micrograms per gram of feces for both pantothenic acid deficient and for pantothenic acid supplemented animals.

Summary. The concentration of riboflavin in the stools of young rats maintained on a riboflavin deficient diet or at intakes of 10 μg or 40μg of this vitamin per day remained constant throughout a test period of 40 days and was essentially the same for all groups.

The total output of riboflavin was dependent upon the quantity of feces excreted. Rats receiving riboflavin but with the caloric intakes restricted to the extent of 50% excreted less total riboflavin than did their *ad libitum* controls.

Addendum. After this paper had been accepted for publication, Schweigert *et al.*[8] reported that cecectomized rats on complete synthetic rations and synthetic rations containing limited amounts of the individual B vitamins grew at the same rate as their controls. They concluded that growing rats on sucrose-containing diets did not depend to any extent on the cecum for the absorption or synthesis of these known or required factors.

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[8] Schweigert, B. B., McIntire, J. M., Henderson, L. M., and Elvehjem, C. A., *Archiv. Biochem.*, 1945, **6**, 403.

15067 P

Vascular and Circulatory Changes in Renal Cortex in the Anuric Crush-Syndrome.

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In this communication, the vascular and circulatory changes in the renal cortex (tissues fixed in formol; preparations stained by Masson's trichrome method) of 4 cases of fatal, so-called renal crush-syndrome[1] are briefly reported. In each of the 4 cases, the subjects had received crushing injuries of the extremities and died 4 to 9 days later following the development of marked oliguria and anuria.

Histologic examination of the kidneys disclosed the following state of the microscopic circulatory structures:

(1) glomerular ischemia or oligaemia.

(2) in some zones, slight congestion of the inter-tubular capillaries with inter-tubular oedema.

(3) (a) enlargement of the afibrillar constituents of the juxta-glomerular apparatus[2,3] and very marked granularity of its cells; eventually degeneration of these cells. (b) increase in number and hypertrophy of the afibrillar (granular and non-granular) cells of the media of the pre-glomerular, and, exceptionally, the interlobular arterioles.

(4) occasionally (in 2 of the 4 cases) the presence of Siegmund's intimal granulomas[4] and parietal fibrinoid thrombi in the interlobular veins.

A more detailed account follows:

Glomerular changes: the erythrocytes in the loops are either absent or scanty; the mesangium (Zimmermann[5]), is unaltered; the loops are filled with coagulated plasma and a few enlarged endothelial cells. The wall of the distended infundibulum sometimes contains an increased number of spindle-shaped cells separated by fine collagen fibrils. The glomerular space is distended by coagulated (by fixation) albuminous material.

The cellular layer of Bowman's capsule is unaltered, but as the result of the distension of the glomerular space, the hypertrophied epithelium of the neck of the proximal convoluted tubule appears incorporated in the glomerular capsule.

An incidental finding in one case is fat embolism of the tufts of slight degree.

The inter-tubular capillaries: the blood circulation between the tubules has been maintained apparently by the collateral circulation of the renal capsule and Ludwig's arterioles, since the inter-tubular capillaries contain erythrocytes; in some places they appear slightly engorged. Inter-tubular oedema is conspicuous except in the cortex corticis. It is accompanied by slight histiocytic reaction and an early deposit of collagenous fibrils.

The juxta-glomerular apparatus: normally, the afibrillar cells of the renal arterioles in man are devoid of granules,[2,6] however, they are abundant in the anuric kidneys associated with crush wounds reported here.

In no other pathologic condition yet examined is the hypertrophy and granularity of the afibrillar constituent of the juxta-glomerular constituent of the juxta-glomerular apparatus so marked. The fuchsinophil (Masson's tri-chrome stain) granules, generally coarse, dissolve gradually (vacuolar complex of Dunihue and Candon[7]). This process as exemplified especially in one case, is associated with intense vacuolation of the afibrillar cells. Pycnosis and shrinkage of the afibrillar cells is sometimes observed.

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[1] Bywaters, E. G. L., and Dible, J. H., *J. Path. and Bact.*, 1942, **54**, III.

[2] Goormaghtigh, N., *Arch. Biol.*, 1932, **43**, 575.

[3] McManus, J. F. A., *Lancet*, 1942, **2**, 394.

[4] Siegmund, H. *Verhandl. Deutsch. Pathol. Gesellsch.*, 1925, **20**, 260.

[5] Zimmermann, K. W., *Z. f. mikr. Anat. Forschg.*, 1933, **32**, 176.

[6] Graef, I., *Am. J. Path.*, 1943, **19**, 121.

[7] Dunihue, F. W., and Candon, B. H., *Arch. Path.*, 1940, **29**, 777.

This afibrillar and granular transformation of the cells of the media of the preglomerular arterioles is a distinctive feature of the anuric kidney. The interlobular arterioles may also be involved. These afibrillar cells are as a rule in marginal position except at the arteriolar bifurcations where they are found immediately under the endothelium. Where this transformation is very extensive, it leads to the almost complete disappearance of the fibrillar muscle cells. The lumen of the arterioles is then wide and filled with stainable serum.

The intimal granulomas (Siegmund)[4] of the interlobar veins, contain histiocytes, large cells of which the origin is not yet determined, spindle cells (muscle and connective tissue cells), red blood cells, blood platelets, fibrin and a hyalin substance. The endothelium is hyperplastic and later undergoes fibrinoid necrosis. The granuloma then becomes a mural, fibrinoid thrombus. The histogenesis of these intimal granulomas has been demonstrated. (Siegmund[4] and Kusama).[8]

Discussion. The vascular changes reported here are very similar to those observed in eclamptic anuria. In such cases Goormaghtigh[9] found that the glomerular tufts were ischaemic but unaltered in their structure, and that there was marked afibrillar transformation of the pre-glomerular arterioles. The granularity of the afibrillar cells was, however, not so pronounced as in the "crush-syndrome" cases. A great similarity exists also with very acute fatal glomerulo-nephritis (Volhard,[10] Goormaghtigh[2]). Moreover this granular transformation of the preglomerular arterioles is the distinctive feature of the ischaemic kidney of the rabbit in the Goldblatt experiment (Goormaghtigh),[11] and, according to this author, plays an important part in the production and liberation of the vasopressive substance formed in increased amount in the ischaemic kidney.[10-12] If this premise is correct, there is reason to believe that in "crush-injury anuria," this vasopressive substance is liberated in excess and causes a persistent spasm at the vascular pole of the glomerular tufts so long as contractile cells persist in the arteriolar wall or in the glomerular tuft.

The concept of the existence of vascular spasm is perhaps corroborated by the presence of the intimal granulomas noted in the interlobular veins. It is well known that these granulomas appear in allergic conditions (Siegmund,[4] Aschoff,[14] Rössle[15]) and the latter, it is generally agreed, are characterized notably by localized, excessive vascular spasms.

As to the cause of the enhanced endocrine vasopressive reaction (Goormaghtigh[13]), of the renal arterioles, local anoxia, associated with the extensive muscular alteration, seems to be the determining factor (cf. the cytology of the ischaemic kidney). Nevertheless the effect on the afibrillar cells of the pathologic glomerular filtrate (Bywaters & Dible[1]) acting via the macula densa (Zimmermann[5]), or via the severely altered tubular cells[1] in contact with the arterioles, must also be considered.

Summary. In 4 instances of fatal, so-called renal crush syndrome in man, study at necropsy disclosed glomerular ischemia or oligemia; enlargement of the juxta-glomerular apparatus with increased granularity of the cells, hyperplasia and hypertrophy of the afibrillar (granular and non-granular) cells of the media of the pre-glomerular arterioles. In 2 of the 4 cases, intimal granulomata and parietal thrombi were found in the interlobular veins. Since similar changes are found in ischemic (Goldblatt) kidneys, in which the author postulated an endocrinologic role for the affected cells, consisting of the liberation of a vasopressor substance, it is suggested that similar liberation of a vasopressor substance occurs in the anuric crush syndrome, causing arteriolar spasm at the vascular pole of the glomerular tufts.

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[8] Kusama, S., *Beitr. path. Anat.*, 1913, **55**, 459.

[9] Goormaghtigh, N., *Bull. Acad. Roy. Med. Belg. VIe. serie*, 1942, **7**, 194.

[10] Volhard, F., *Handb. innere Mediz. 2e. aufl.*, **6**, Berlin, J. Springer, 1931.

[11] Goormaghtigh, N., PROC. SOC. EXP. BIOL. AND MED., 1939, **43**, 575.

[12] Goormaghtigh, N., and Grimson, K. S., PROC. SOC. EXP. BIOL. AND MED., 1939, **42**, 227.

[13] Goormaghtigh, N., *La fonction endocrine des artérioles rénales*, Librairie Fonteyn, Louvain, 1944.

[14] Aschoff, L. *Verhandl. Deutsch. Path. Gesellsch.*, 1925, **20**, 272 (discuss. Siegmund).

[15] Rössle, R., *Virchow's Arch.*, 1933, **288**, 780.

15068 P

Potentiating Effect of Anaphylactic and Histamine Shock upon Herpes Simplex Virus Infection in Rabbits.*

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A rabbit which had been inoculated intramuscularly and again intracerebrally with herpes simplex virus suspension with no apparent infection was, after a period of 6 months, sensitized to egg-white and thrown into anaphylactic shock. On the seventh day following the shock, the first symptoms of herpetic encephalomyelitis appeared. On the ninth day the animal was moribund and was dispatched.

Protocol—Rabbit G 126.

7/30/44	Inoculated with 1 cc 10% mousebrain suspension of $H.F._{12}$ virus into quadriceps femoris muscle of right leg.
7/31 to 8/28	Daily temperatures normal.
8/28	Intracranial inoculation with 0.2 cc 10% suspension of same virus. No illness followed this inoculation.
1/23/45	5 cc egg-white injected subcutaneously into right hind leg.
1/25	2 cc 10% egg-white in distilled water intravenously.
2/ 6	0.75 cc egg-white intravenously; severe anaphylactic shock ensued.
2/ 8	Animal well.
2/ 9	No change.
2/10	No change.
2/13	Animal depressed RT. 104.9.
2/14	Rectal temperature 106.2. Severe opisthotomy, grinding of teeth, twitching of facial muscles, excessive salivation, head nystagmus, excitement noted.
2/15	Condition worse. Animal killed. Autopsy revealed extensive encephalomyelitis with typical herpetic inclusion bodies.

Trauma, intracerebral injections of irritating solutions, and extensive subcutaneous inflammation have been shown by Le Févre de Arric and Millet[1] to increase the susceptibility of rabbits to intravenous inoculation with Herpes simplex virus.

The history of rabbit G 126 and the reports in the literature, cited above, have led us to the following experiments which were designed to test the potentiating effect of shock upon susceptibility to intravenous inoculation with the virus of Herpes simplex. Histamine shock has been substituted for anaphylactic shock.

Experiment I. Six young adult rabbits (2.5-3.0 kg) were inoculated intravenously with 1 cc of the supernatant of a centrifuged 10% infected mouse brain suspension in Locke's solution.‡ Three of the animals (G 136, 137, 138) had been subjected to severe anaphylactoid shock 10 minutes previously by the intravenous injection of 2.75 mg of histamine diphosphate. The other 3 animals (G 139, 140, 141) were given no other treatment. Of the 3 animals in which the herpetic inoculation was preceded by shock, 2 (G 137, 138) developed severe encephalomyelitis on the seventh day and were killed when moribund. The third, becoming ill on the eighth day, showed less marked but definite signs of encephalitis and none of myelitis. The 3 animals were autopsied and the presence of typical herpetic lesions noted in the brain and in rabbits G 137, 138, in the spinal cord. Bio-assays of the latter 2 animals revealed virus in the midbrains and spinal cords. Of the 3 rabbits inoculated without the preceding shock, none became ill nor showed herpetic lesions at autopsy 21 days later.

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* Aided by a grant from the National Foundation for Infantile Paralysis.

[1] de Févre de Arric, and Millet, M., *C. R. Soc. Biol.*, Paris, 1925, **93**, 45; 1926, **94**, 782.

† Bio-assays of these 2 animals revealed virus in the midbrains and spinal cords of each; the livers, kidneys, spleens, and adrenals were negative. The virus which was recovered had, after the second mouse passage, the same m.l.d.$_{100}$ as the original infective dose.

‡ The Herpes simplex virus used in these experiments was maintained by intracerebral mouse to mouse passage. The m.l.d.$_{100}$ was 0.03 cc of a 1/10000 suspension of the brain.

Experiment II. Seven young adult rabbits (2.9-3.5 kg) were inoculated with 1 cc of the supernatant from a 10% suspension of infected mouse brain in Locke's solution. Ten to 15 minutes preceding this inoculation, 4 of the animals (G 151, 152, 153, 154) were given a severe anaphylactoid shock by intravenous injection of 2.75 mg histamine diphosphate. By the seventh day, these animals had all shown signs of herpetic encephalitis. At autopsy widespread lesions were found in the nervous system. Of the 3 animals not shocked previous to the herpetic inoculation (G 148, 149, 150) 2 showed neither signs nor lesions of the disease while one developed a mild encephalitis on the seventh day. At autopsy lesions were found in the midbrain and thalamus of the latter animal.

Discussion and Summary. Intravenous infection of rabbits with neurotropic herpes simplex virus is only occasionally possible according to Le Févre de Arric and Millet,[1] Doerr and Vöctung,[2] and Van Rooyen and Rhodes.[3] This fact is borne out by our control series. The fact that all experimental animals in the series here reported were infected clearly reveals the potentiating effect of histamine shock on susceptibility to the virus. It would seem, from the first animal reported, that the effect is related to a common denominator of histamine and true anaphylactic shock.

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[2] Doerr, R., and Vöchtung, R., *Rev. gén. Ophthal.*, Paris, 1920, **77**, 372.

[3] Van Rooyen, C. E., and Rhodes, A. J., *Virus Diseases of Man*, London, 1940.

15069

Effects of Sulfonamides on Chick Brain Tissue Cultivated *In vitro*.*

JOHN B. DEC. SAUNDERS AND WEBB HAYMAKER. (Introduced by Chauncey D. Leake.)

From the Department of Anatomy, University of California Medical School, San Francisco, Calif.

Although it has become an established practice to apply certain sulfonamide compounds to brain exposed by craniotomy or as a result of war wounds, little is known of the effect of these drugs on brain elements. In an effort to establish a basis for comparison of the toxicity of sulfonamides, brain tissue of the chick was cultivated in plasma containing the following compounds: (1) sulfanilamide, (2) sulfapyridine, (3) sulfathiazole, (4) sulfaguanidine, (5) sulfadiazine, (6) nicotinyl sulfanilamide, (7) sulfapyrazine, (8) succinyl sulfanilamide, (9) succinyl sulfathiazole, (10) sodium sulfapyridine, (11) sodium sulfathiazole, and (12) sodium sulfadiazine.

Methods. In order to avoid the introduction of variables, the procedure of cultivation was reduced to the simplest possible elements. Brain tissue was excised from 8-day old chick embryos and placed in a Petri dish containing Tyrode's solution. The meninges were care-

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* The work described in this paper was done under a contract recommended by the Committee of Medical Research between the Office of Scientific Research and Development and the University of California.

fully dissected off and the brain cut into fragments of approximately 0.5 sq. mm. The fragments were quickly transferred for planting to Carrel D-3 flasks containing a medium of chicken plasma.

The plasma was obtained by bleeding roosters from the carotid arteries without anaesthetic. The blood was drawn off into chilled paraffined tubes, then centrifuged for 20 minutes, whereupon the supernatant plasma was pipetted off into unparaffined tubes and stored in the refrigerator.

The method of planting was standardized. Into each Carrel flask was introduced 1.5 cc of plasma. The plasma employed at each initial planting was fresh (under 2 days), and in each instance the remainder of the identical plasma was reserved for patching. Approximately 5 plants were made per Carrel flask and as coagulation of the medium occurred after the introduction of the tissue, the use of embryonic extract was avoided. The flasks were then stoppered and incubated at 38°C. On the average of every second day the cultures were removed from the incubator, washed in Tyrode's solution for a period of 5 to 10 minutes, the fluid decanted, and the culture patched with 0.5 cc of the same plasma used initially. When digestion of plasma proceeded with unusual rapidity, patching was done daily. Explants from subcultures were not done since it was felt that the conditions obtaining in the local application of sulfonamides in the treatment of wounds of the brain could best be met by maintaining the original plant over the prescribed period.

In the experimental cultures, the same technique was employed except that a weighed amount of a sulfonamide was added to the plasma used for planting and patching. All of the sulfonamides employed were weighed out, placed in tubes and sterilized by dry heat in an oven at 140°C for 3 hours. To each tube of the sterilized compound the requisite amount of plasma was added to obtain the concentration desired for both planting and patching, the concentration extending with each drug, from below to somewhat above its saturation point in plasma maintained at 38°C. With each test series, collateral controls, grown in plasma from the same source, were included.

Of the several available methods for measurement of growth in tissue cultures, one similar to that of Ebeling[1] was adopted. Immediately after planting, the cultures were projected by means of a projectoscope modified for this purpose, identified, and the initial plant outlined on paper. On the sixth day, at the conclusion of the experiment, the flask was again projected and the area of extension traced. The surface areas of the tracings of both plant and growth were then measured with a planimeter. From the total area of the plant and its growth, derived by addition of the areas of all cultures comprising a number of a series, the ratio of plant to growth was established. The projection was done at high magnification, 59 diameters, in order to exclude all areas of plasma uninvaded and to furnish a means of outlining the extension of neurones and neuroepithelium. Areas of wandering cells were excluded. After tracing the projected growth on paper, the cultures were viewed microscopically and, where necessary, appropriate modifications were introduced into the traced outline.

From preliminary work it was found unnecessary to make adjustments in pH of the plasma, as a rich growth could be obtained in a test period of 6 days with the routine described above. The factor of pH of the sulfonamides had, however, to be taken into consideration in this study. The first step was to determine the pH of saturated solutions of sulfonamides in plasma at 38°C. This was done by means of a standard Berckman pH meter. The pH of the sulfonamides was found to range from 6.75 to 10.20, most of them between 7.15 and 7.57 (Table I).

The next step was to set up an experiment to test the effect of pH alone on brain cultures. The method of culture was identical to that employed in the foregoing sulfonamide studies. The plants were irrigated twice daily with a solution in the desired range of pH. The solutions were prepared as follows: To "glucosal" (Tyrode's solution without the addition of sodium carbonate) from which

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[1] Ebeling, A. H., *J. Exp. Med.*, 1921, **34**, 231.

TABLE I. Data on Growth of Chick Brain Cultures in Plasma to Which Sulfonamides Were Added in Various Concentration. The figures are those obtained 6 days after planting.

Sulfonamide	Approx. solubil. in plasma, mg/cc	pH of sulfonam. at satur. in plasma, 38°C	Range of concentr. of sulfonam. tested, mg/cc	No. cul. grown, incl. controls	Growth ratio of controls	Conc. up to which growth of experimentals was optimal	Corresp. growth ratio	Min. concentr. proving toxic	Corresp. growth ratio
Sulfadiazine	0.4	7.15	0.01- 2.0	283	1: 6.5	2.0	1: 8.3	†	†
Succinyl sulfathiazole	2.0	7.48	0.10- 6.0	229	1: 8.1	2.0	1:15.4	†	†
Sulfapyrazine	1.0	7.70	0.20- 2.0	145	1: 4.0	0.5	1: 5.5	2.0	1:4.3
Sulfapyridine	0.6	7.55	0.10- 1.0	208	1: 4.5	0.3	1: 5.3	1.0	1:4.0
Sulfaguanidine	1.0	7.57	0.20- 2.0	147	1: 3.0	1.0	1: 4.0	2.0	1:2.5
Sulfathiazole	0.7	7.20	0.20- 3.0	175	1: 2.6	0.5	1: 2.8	1.0	1:2.0
Succinyl sulfanilamide	1.0	6.75	0.20- 6.0	136	1:13.7	0.2	1:10.2	0.5	1:5.5
Nicotinyl sulfanilamide	0.8	7.57	0.02- 1.0	234	1: 4.0	0.02	1: 2.1	0.02	1:2.1
Sulfanilamide	9.0	7.50	2.00-12.0	328	1: 2.6	2.0	1: 3.4	4.0	1:1.6
Sodium sulfadiazine	900.0	9.20	0.20- 6.0*	220	1: 5.1	0.5	1: 7.6	6.0	1:2.2
Sodium sulfathiazole	800.0	9.20	0.10- 6.0*	226	1: 5.4	1.0	1: 5.8	3.0	1:2.2
Sodium sulfapyridine	500.0	10.20	0.30- 2.0*	75	1: 5.0	0.3	1: 2.5	0.3	1:2.5

* Owing to the high degree of solubility of these drugs and the unlikelihood of their local use at saturation it was deemed advisable to test these compounds within a range corresponding to the others.

† Cultures grew well at all concentrations tested. Concentrations higher than 2.0 mg/cc could not be tested because crystals of drug obscured visibility.

acid sodium phosphate had been withheld, was added an indicator (phenol red or thymol blue) and then, after sterilization, the solution was adjusted to the desired pH by the addition of the necessary amounts of sterile standard sodium hydroxide solution. In this manner pH concentrations ranging from 5.0 to 9.6 were obtained.

Owing to the buffering power of relatively small amounts of plasma, there was considerable difficulty in maintaining the pH of the cultures to the desired level. This was particularly true of efforts made to reduce the pH of cultures to the acid side even though as much as 1 to 2 liters were employed as the irrigant. Taking this condition of experiment into consideration it may be said that with partial maintenance of the pH at the desired levels the effect of pH on brain cultures was as follows: at pH 5.0, 5.3 and 6.6 there was no growth; at 7.0 a slight outgrowth of neurones as well as moderate neuroglial extension and considerable vacuolization of all cells; at 7.4, and 7.8, excellent growth; at 8.0, moderate to slight growth; at 8.2 to 8.5, slight growth with early vacuolization of cells; and at 9.0 and 9.6 very slight growth with early and severe vacuolization. Thus, the range of pH most favorable to growth of brain in culture was between 7.4 and 7.8. In a parallel series of heart cultures of 8-day-old chick embryos, 297 cultures in all, which were planted in the same flasks as the brain, the growth was also most prolific in this range of pH but there was moderate growth, with evidence of early degeneration, at pH of 6.6. to 7.4 and from 8.0 to 9.6.

From these figures it is apparent that the pH of most of the sulfonamides was well within innocuous limits, and it seems logical to conclude that where the levels of pH were slightly above or slightly below the ideal range the effects were obviated by the buffering power of the plasma. Hence these preliminary experiments seem to warrant the conclusion that the effect exerted by sulfonamides on brain cultures were due to innate properties of the drugs, and not to their pH.

Results. Data on the growth of chick brain cultures in plasma to which sulfonamides were added are given in Table I. Except for the sodium salts, which are considered separately, the sulfonamides are listed in order of increasing toxicity. Sulfadiazine was the most satisfactory of the group. Cultures grew well at all concentrations tested, even up to five times saturation in plasma. Planimeter measurements suggested that the drug had a stimulating action at higher concentrations. Sulfadiazine was the least soluble of the series (Table I) and its pH in plasma was somewhat below the optimum. Next in order came succinyl sulfathiazole and sulfapyrazine, the growths equaling or somewhat exceeding those of the controls at all concentrations tested. Then came sulfapyridine and sulfaguanidine: cultures containing these drugs grew well up to saturation and somewhat beyond; only at about double the saturation point was the growth less than in the controls. Sulfathiazole, next on the list, when at half saturation had no inhibiting influence on growth, but at saturation and beyond there was early and severe degeneration of nerve cells. Succinyl and nicotinyl sulfanilamide and sulfanilamide itself fall into much the same class inasmuch as they are toxic at approximately half saturation. Of these, succinyl sulfanilamide has a selective effect at concentrations in excess of saturation, producing dissolution of the initially migrating nerve cells and severe vacuolization of neuroglial and wandering cells. Growth of cultures to which sulfanilamide were added was satisfactory up to half saturation; above saturation there was no growth whatever.

The sodium salts, because of their high solubility, were tested over a limited range in the lower concentrations. Judging these compounds at like levels of percentage concentration of saturation, they fall into the following order of increasing toxicity: sodium sulfadiazine, sodium sulfathiazole and sodium sulfapyridine. Cultures containing sodium sulfadiazine and sodium sulfathiazole, respectively, grew as well as the controls up to concentration of approximated 2.0 mg/cc.

Conclusions. Brain of 8-day-old chick embryos cultivated *in vitro* in plasma to which sulfonamides in various concentrations were added, responded differently in accordance with the sulfonamide tested. Cultures containing sulfadiazine and succinyl sulfathiazole grew better than the controls in all concentrations tested. The growths in sulfapyrazine and sulfapyridine equalled those of the controls. The other sulfonamides showed varying degrees of toxicity. Preliminary experiments suggested strongly that the toxicity is to be ascribed to inherent properties of the drugs, not their pH.

The authors wish to express their indebtedness to Josephine Campbell, Messrs. Frank Southard, and Norman W. Karr for their technical assistance in the carrying out of this problem and to Professor Chauncey D. Leake for valuable advice.

15070 P

Effect of Digitalization on the Coagulation Time in Man.

L. SOKOLOFF AND M. I. FERRER. (Introduced by A. C. DeGraff.)

From the Department of Therapeutics, New York University, College of Medicine.

Within recent years a number of communications concerning the influence of digitalis on the coagulability of the blood has appeared. Tanaka[1] found that parenterally administered strophanthin shortens the blood coagulation time of dogs and Werch[2] reported that digifolin exerts a similar effect in rabbits. Macht[3] observed that several of the glycosides shorten the coagulation time of cat plasma to the order of 1/10 to 1/5 of its initial value *in vivo* and also act *in vitro*. Recently Massie *et al.*[4] stated that digitalis leaf in clinical doses shortens the coagulation time of the whole blood of man. Digitalis has also been reported to make heparin less effective in prolonging the blood coagulation time of man and dogs.[5] As a result, several clinical notes have appeared suggesting that digitalis therapy may favor the development of thrombotic disease.

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[1] Tanaka, H., *Okayama-Igakkai-Zasshi,* 1928, **40,** 1826.

[2] Werch, S. C., *Quart. Bull. Northwestern U. M. Sch.,* 1943, **17,** 50.

[3] Macht, D. I., *Ann. Int. Med.,* 1943, **18,** 772.

Selection of Material. Ten patients exhibiting some degree of heart failure were studied. Because an observation period of several days was required before specific therapy was initiated, these patients were necessarily not too seriously ill. Because admixture of tissue juices exerts a thromboplastic influence on the coagulation process, patients who did not have a sufficient number of easily penetrable veins were not accepted as subjects. Other medications which have been considered to influence coagulability of the blood were withheld, including salicylates, aminophylline and mercurial diuretics. In one instance (S.W.) a single dose of mercupurin was administered after initiation of digitalis therapy. Since mercurials have been considered to increase coagulability of the blood[6] and since no shortening of the coagulation time occurred, we feel justified in including this case. The coagulation time was determined for at least 4 days before onset of digitalization and for a comparable period during initiation of this therapy. The rapid method of Eggleston was followed. In 9 cases the U.S.P. XII leaf preparation and in one case the purified glycoside digitoxin was used. Six patients were digitalized to the point of minor toxicity, 5 receiving the whole leaf, and one receiving digitoxin. Of the remaining 4 patients, all received digitalis leaf. Two received 15 and 22 U.S.P. XII digitalis units, respectively, in 5 days. The third patient received 21 units in 4 days, the fourth 13 units in 6 days. All 10 patients can be considered adequately digitalized since a full therapeutic effect was obtained in each instance and the average initial digitalizing dose with this preparation is 12 digitalis units.

Method. The method was essentially that of Lee and White. Particular attention was paid to performing the venepuncture expeditiously; unless this was accomplished directly, the specimen was rejected. An 18-gauge needle was used and the tourniquet removed as soon as the antecubital vein was entered. Three test tubes, 14 mm x 100 mm, were jacketed in thermos-jug water baths between 35° and 40°C. All tubes were cleaned daily with chromic acid cleaning solution. Just before use, they were rinsed with sterile, physiological saline solution, as were the needles and syringes. Three cc of blood were withdrawn into a 5 cc syringe and 1 cc transferred gently to each of the test tubes. The samples were allowed to stand for 4 minutes. The first tube was then tilted every half minute until the blood failed to flow down the side. The second and finally the third were similarly tilted. The time at which the blood ceased to flow in the third was considered the coagulation time.

Results. A considerable variation in the coagulation time was observed from day to day in several of the patients. This reflects in part the intrinsic coagulability of the blood, and in part the essentially crude procedure through which many extrinsic variables operate, particularly the agitation against the foreign surface of the container. Forty-seven determinations were made before and 42 following onset of digitalization. In 4 cases there was some shortening of the average coagulation time following initiation of the therapy; in 5 cases there was a slight prolongation; and in one case, no change at all. The average time for all determinations before digitalization was 9.45 minutes (S.D. = ±1.55 for 46 degrees of freedom) and 9.50 minutes (S.D. = ±1.41 for 41 degrees of freedom) during digitalization. The population standard deviation, which indicates the reproducibility of the method is ±1.18 minutes with 69 degrees of freedom. An analysis of variance was computed: that due to digitalis was 0.060; that between patients was 8.762; that reflecting the interaction (digitalis × patients) was 2.089; the error within patients was 1.386. It is evident that the clotting times of individual patients differed significantly among themselves regardless of therapy. However, the variance due to treatment with digitalis is considerably less than that due to experimental error. Consequently, these experiments fail to support the hypothesis that oral digitalization increases the coagulability of the blood as measured by this technic.

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[4] Massie, E., Stillerman, H. S., Wright, C-S, and Minnich, V., *Arch. Int. Med.*, 1944, **74**, 172.

[5] de Takats, G., Trump, R. A., and Gilbert, N. C., *J. A. M. A.*, 1944, **125**, 840.

[6] a. Wiebel, H., *Klin. Wchnschr.*, 1940, **19**, 1013; b. Link, K. P., *Harvey Lecture*, 1943-44, **39**, 162.

Summary. Whole blood coagulation times were determined before and during oral digitalis therapy of 10 patients. No significant alteration of coagulability as so measured was observed.

15071

The Anti-Histamine Effect of Imidazol.*

HAROLD C. MORRIS AND CARL A. DRAGSTEDT.

From the Department of Pharmacology, Northwestern University Medical School, Chicago, Ill.

From a study of several compounds (histidine, arginine, cysteine, etc.) which inhibit the action of histamine upon smooth muscle structures, Ackermann and Wasmuth[1] postulated that histamine owes its pharmacological activity to its =NH imine group, and to its NH_2 group, the former acting as an anchoring group with the chemical receptors of the smooth muscle, and the latter as the stimulating agent. The inhibiting compounds, having imine but no amine radicals, bind the chemical receptors, have no pharmacological effects themselves, but block the effects of histamine. Rocha e Silva[2] confirmed such a view by showing that various histamine compounds in which the amine radicals were masked, were inactive themselves but acted as blocking agents against histamine.

On the basis of this theory one could expect that imidazol (Glyoxalin) would possess antihistamine properties because it presents a free imine radical.

Imidazol was therefore tested as to its counteracting effect against the stimulating action of histamine upon the surviving guinea pig intestinal strip and its vaso-depressor action in anesthetized cats. Following Rocha e Silva's technic, histamine was added to the bath in a series of concentrations to determine the dose which produced a marked but submaximal contraction. Imidazol (Eastman), neutralized, was then added to the bath in increasing amounts, noting the effect of each increment upon the response to the test dose of histamine. In a series of experiments it was found that imidazol would block the effects of histamine when present in a ratio of 1500 to 1. That the imidazol did not act by damaging the intestinal strip was indicated by the fact that there was prompt recovery of histamine responsiveness on washing. In the cat, the intravenous injection of imidazol (1 to 128 mg per kilo) diminished, but did not obliterate the depressor effect of histamine. Doses of 1 mg per kilo reduced the effect of histamine about 25%, and doses of 128 mg per kilo about 60%. The latter dose approximates the maximally tolerated dose.

Summary. Strong concentrations of imidazol can block the effect of histamine upon the guinea pig intestine, and large doses can decrease the vasodepressor effect of histamine in the cat, but the amounts required are so great as to offer no promise of clinical or laboratory usefulness.

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* This investigation has been made with the assistance of a grant from the Clara A. Abbott Fund of Northwestern University.

[1] Ackermann, D., and Wasmuth, W., *Z. f. Physiol. Chem.*, 1939, **258**, 28.

[2] Rocha e Silva, M., *J. Pharmacol. and Exp. Therap.*, 1944, **80**, 399.

ANNUAL REPORT OF SECRETARY

April 1, 1944-April 1, 1945

Election of Officers.

President Carlson appointed Doctors Greenwald, Mettler, Milhorat and Seegal as tellers. These tellers report as follows:

For President:	*A. B. Hastings*	565	votes
	C. J. Wiggers	415	"
For Vice-Pres.:	*E. M. K. Geiling*	530	"
	H. M. Smith	436	"
For Sec'y-Treas.:	*A. J. Goldforb*	946	"
For Council members at large	*C. A. Dragstedt*	596	"
	G. R. Cowgill	517	"
	H. B. Lewis	506	"
	H. A. Mattill	277	"

The italicized names are declared elected.

Finances.

It has been the policy of the Council to plan for a minimal annual surplus (to meet possible emergencies). This was done by progressive reduction (far below cost) of publication charges. Nevertheless there was, for the past fiscal year, the largest annual surplus in the history of the Society, *viz.,* $6,946.20. (For details see the report of the Treasurer.) This surplus was due primarily to the large number of subscriptions purchased by the U. S. Government for the medical services in the armed forces here and abroad.

Because of this large surplus the Council authorized the following, *for the coming fiscal year:*

I. a. Charge for excess space to be reduced from 13% of cost to no cost.

b. Illustrations reduced from 50% of cost to 25% of cost.

II. A small increase in salary to Miss McCabe.

III. The PROCEEDINGS to be mailed to pre-war subscribers not otherwise provided for by the Rockefeller Foundation and other agencies, and to members, in the allied war zones, without charge.

Finance Committee.

The Society is greatly indebted to the Finance Committee for its painstaking and most competent care of the investments of the Society. Dr. Lambert obtained the advice of the investment department of the Rockefeller Foundation. Dr. Leake obtained the advice of the investment department of his university. The Society is also much indebted to Mr. H. G. Friedman of the General American Investors Corporation, who has continuously given his expert advice, to Mr. Leon Leighton, who has advised on legal phases of our guaranteed mortgages and to Mr. William Girden for help on the non-legal phases of this problem. It is needless to say they served without compensation.

The Southwest Section.

The members from Texas and adjoining regions requested that the name of their Section be changed to the Southwestern Section, and to include the adjoining states. The Council approved.

No meetings of the Council and the National Committees were held, in conformity with Government request. It is hoped that these meetings may be possible next year.

Editorial Matters.

The Society is greatly indebted to the Editors for the splendid service they have rendered in these arduous days. It may be of interest to note that there has been very little reduction in the number of manuscripts accepted during the past war period. The standards of acceptance have been progressively raised. We regret the unavoidable delays in publication.

The new policy provides for publication of complete manuscripts up to ten pages in length, and in exceptional cases more space may be permitted. For the period from October 1944 to April 1945 inclusive, there were 275 articles of which 17% were preliminary, 83% complete.

Length of articles is summarized as follows:

Length in pages	*Preliminary No. of Articles*	*Complete No. of Articles*
1	11	7
1½	19	31
2	11	57
2½	2	46
3	3	31
4	0	36
5	0	15
6	0	3
7	0	3

The Society is much indebted to Dr. Emil Baumann for his continued and excellent indices for each volume of the PROCEEDINGS.

Newly Elected Members

M. A. Andersch
W. Andrew
L. Anigstein
C. P. Barnum, Jr.
D. L. Bassett
K. H. Beyer, Jr.
B. Campbell
G. L. Cantoni
G. F. Cartland
C. Chagas
W. D. Collings
T. C. Daniels
K. Dobriner
V. A. Drill
F. W. Dunihue
L. G. Farmer
G. J. Fashena
O. G. Fitzhugh
G. F. Forster
A. J. Glazko
L. Gross
A. J. Haagen-Smit
T. R. Harrison
L. W. Hazleton
W. F. Hollander
C. W. Hooker
M. H. Kuizenga
C. M. MacLeod
L. M. Meyer
B. B. Morgan
K. A. Oster
D. M. Pace
R. F. Pitts
A. A. Plentl
R. D. Reid
S. Rothman
H. F. Schott
H. Schwarz
F. Schlenk
A. Segaloff
F. J. M. Sichel
R. W. Sperry
H. C. Stoerk
L. Thomas
H. C. Tidwell
C. Torda
H. F. Traut
C. R. Treadwell
T. Tung
A. Wikler
R. J. Winzler
H. G. Wood
L. D. Wright
L. Zechmeister

Emeritus Members. The following members qualified for emeritus membership and recommended by their Sections have been elected to emeritus membership: Doctors M. S. Dooley, G. B. Roth, L. Florence, F. H. Scott, R. M. Yerkes, and A. W. Walker.

Deaths of Members. The Council records with sorrow the death of the following members: Doctors P. J. Beard, R. K. Brewer, M. H. Dawson, A. T. Henrici, W. H. Howell, L. A. Julianelle, I. J. Kligler, C. B. Lipman, L. S. Palmer, F. W. Schlutz, T. A. Storey, and C. C. Young.

Gifts. The thanks of the Society are due the following members for the gift of back numbers of the PROCEEDINGS: Doctors A. P. Briggs, M. B. Cohen, H. G. Day, F. E. Eberson, W. Goldring, P. A. Kober, M. Lipman, A. Marrazzi, M. S. Nichols, W. L. Palmer, I. H. Perry, E. D. Plass, W. H. Seegers, O. H. Wangensteen; and to the following libraries: Abbott Laboratories, Hoffman LaRoche, Inc., State University of Iowa, Montefiore Hospital, National Institute of Health, Northwestern University, Squibb Institute, and Washington University.

PAST OFFICERS

Date	*President*	*Vice-President*	*Secretary*	*Treasurer*
1903-04	S. J. Meltzer	W. H. Park	W. J. Gies	G. N. Calkins
1904-05	S. J. Meltzer	J. Ewing	” ”	” ”
1905-06	E. B. Wilson	E. K. Dunham	” ”	” ”
1906-07	S. Flexner	E. K. Dunham	” ”	” ”
1907-08	S. Flexner	T. H. Morgan	” ”	” ”
1908-09	F. S. Lee	T. H. Morgan	” ”	G. Lusk
1909-10	F. S. Lee	W. J. Gies	E. L. Opie	” ”
1910-11	T. H. Morgan	W. J. Gies	” ”	” ”
1911-12	T. H. Morgan	P. A. Levene	G. B. Wallace	” ”
1912-13	J. Ewing	P. A. Levene	” ”	C. Norris
1913-14	J. Ewing	C. W. Field	H. C. Jackson	” ”
1914-15	G. Lusk	W. J. Gies	” ”	J. R. Murlin
1915-16	G. Lusk	G. N. Calkins	H. C. Jackson	
1916-17	J. Loeb	W. J. Gies	” ”	
1917-19	W. J. Gies	J. Auer	” ”	
1919-21	G. N. Calkins	G. B. Wallace	” ”	
1921-23	G. B. Wallace	J. W. Jobling	” ”	
1923-24	H. C. Jackson	J. W. Jobling	V. C. Myers	
1924-25	H. C. Jackson	J. W. Jobling	A. J. Goldforb	
1925-27	J. W. Jobling	S. R. Benedict	” ”	
1927-29	S. R. Benedict	P. Rous	” ”	
1929-30	P. Rous	D. Marine	” ”	
1930-31	P. Rous	D. J. Edwards	” ”	
1931-32	D. J. Edwards	A. R. Dochez	” ”	
1932-34	A. R. Dochez	E. L. Opie	” ”	
1934-36	E. L. Opie	P. E. Smith	” ”	
1936-37	P. E. Smith	E. F. DuBois	” ”	

1937-39	H. S. Gasser	J. T. Wearn	A. J. Goldforb
1939-40	J. T. Wearn	C. D. Leake	" "
1940-41	J. T. Wearn	C. D. Leake	" "
1941-42	W. deB. MacNider	C. H. Danforth	" "
1942-43	W. deB. MacNider	C. H. Danforth	" "
1943-44	A. J. Carlson	A. B. Hastings	" "
1944-45	A. J. Carlson	A. B. Hastings	" "

SECTIONAL MEETINGS AND MEMBERSHIP

Cleveland, Ohio

Chairman: R. F. Parker Secretary: F. Mautz Members: 49
Meetings: Western Reserve University, October 13, 1944
December 8, 1944
February 9, 1945
April 13, 1945

District of Columbia

Chairman: Henry Stevens Secretary: R. A. Cutting Members: 83
Meetings: George Washington University, December 7, 1944
February 1, 1945
April 5, 1945
Y. W. C. A., Washington, D. C., June 5, 1945

Illinois

Chairman: W. E. Windle Secretary: L. V. Domm Members: 170
Meetings: University of Chicago, February 20, 1945
May 15, 1945

Iowa

Chairman: E. D. Warner Secretary: E. L. DeGowin Members: 40
Meetings: State University of Iowa, November 28, 1944
February 22, 1945
May 8, 1945
May 29, 1945

Minnesota

Chairman: R. G. Green Secretary: W. D. Armstrong Members: 48
Meetings: University of Minnesota, October 25, 1944
November 29, 1944
January 31, 1945
February 28, 1945
May 23, 1945

Missouri

Chairman: L. R. Jones Secretary: C. F. Cori Members: 59
Meetings: Washington University, May 9, 1945

New York

Chairman: I. Greenwald Secretary: W. M. Copenhaver Members: 487
Meetings: New York Academy of Medicine, December 13, 1944
February 14, 1945
May 23, 1945

Pacific Coast

Chairman: P. J. Hanzlik Secretary: M. Kleiber Members: 118
Meetings: University of California, August 26, 1944
Stanford University, October 25, 1944
University of California, December 16, 1944
Stanford University, April 28, 1945

Peiping, China

No report on Peiping Section.

Rocky Mountain

Chairman: L. P. Gebhardt Secretary: A. G. Wedum Members: 29
Meetings: Colorado State College, December 15, 1944
University of Colorado, March 9, 1945
University of Utah, May 26, 1945

Southern

Chairman: A. G. Eaton Secretary: R. G. Smith Members: 45
Meetings: Tulane University, January 12, 1945
Louisiana State University, May 25, 1945

Southern California

Chairman: A. P. Hoyt Secretary: A. M. Schechtman Members: 46
Meetings: University of Southern California, November 30, 1944
California Institute of Technology, March 1, 1945

Southwestern

Chairman: J. A. Greene Secretary: D. Slaughter Members: 37
Meetings: Southwestern Medical College, January 27, 1945
Houston, Texas, June 9, 1945

Western New York

Acting Chairman: A. H. Hegnauer Secretary: A. H. Hegnauer Members: 67

Wisconsin

Chairman: A. L. Tatum Secretary: L. E. Casida Members: 46

MEMBERSHIP

Members, March 31, 1944	1774	
Elected during year	120	
		1894
Resignations	10	
Arrears	2	
Deaths	10	
		22
Total Membership, March 31, 1945		1872

Membership:	1935	1945
	1266	1872

Subscriptions, March 31, 1945	1264

ANNUAL REPORT OF TREASURER

April 1, 1944-April 1, 1945

The committee appointed by President Carlson to audit the accounts of the Treasurer of the Society for Experimental Biology and Medicine has had the books and records examined by Alexander Dolowitz, Certified Public Accountant. The audit report of Mr. Dolowitz is attached and is accepted by the committee as a true statement of the Treasurer's account.

(Signed) George B. Wallace
Joseph C. Hinsey
Aura E. Severinghaus

Statement of Income and Disbursements

April 1, 1944 to March 31, 1945

INCOME

Dues		$ 6,681.70	
Subscriptions	$10,798.38		
Reprints	1,782.25		
Excess Space	431.70		
Cuts	307.52		
Changes	26.58		
Back Numbers	597.30		
		13,943.73	
Interest		2.03	
Miscellaneous		2.34	
Total Income			$20,629.80

DISBURSEMENTS

Office Supplies, Telephone and Postage		$ 646.70	
Cost of Printing	$ 7,414.02		
Cost of Reprints	1,983.06		
Cost of Cuts	655.29		
Storage and Insurance	84.81		
Refunds	4.25		
		10,141.43	
Salaries		2,806.73	
Miscellaneous		88.74	
Total Disbursements			$13,683.60
Excess of Income Over Disbursements			$ 6,946.20

STATEMENT OF ASSETS AND LIABILITIES

AS OF MARCH 31, 1945

(Includes Surplus Fund)

Assets

Cash in Banks	$13,155.08	
Investments—Surplus Fund	24,438.30	
Accounts Receivable	1,055.63	
Total Assets		$38,649.01

Liabilities

Accounts Payable	$ 16.62	
N. Y. Section	185.22	
Withholding Taxes	126.50	
Total Liabilities		328.34
Net Worth		$38,320.67

Endowment Fund

Guaranteed Mortgages	$ 5,273.56	
Bowery Savings Bank	1,929.47	
U. S. Government Bonds	11,100.00	
R. R. Federal Savings and Loan Assn.	77.26	
Industrial Bonds	2,923.62	
Total		$21,303.91

MEMBERS' LIST

Abels, J. C.	Memorial Hosp., N. Y.
Abramson, D. I.	May Inst. Med. Research Cincinnati
Abramson, H. A.	Coll. Phys. and Surg., N. Y.
Abt, Arthur F.	Northwestern Univ.
Acevedo, D.	Univ. de San Marcos, Peru
Adams, A. Elizabeth	Mount Holyoke Coll.
Addis, Thomas	Stanford Univ. Med.
Adlersberg, D.	Beth Israel Hosp., N. Y.
Adolph, E. F.	Univ. of Rochester Med.
Adolph, W. H.	Cornell Univ.
Adriani, J.	Louisiana State Univ.
Albaum, H. G.	Edgewood, Md.
Alexander, Harry L.	Washington Univ.
Allen, Bennet M.	Univ. of Calif., L. A.
Allen, Frank W.	Univ. of Calif.
Allen, J. G.	Univ. of Chicago
Allen, William F.	Univ. of Oregon
Alles, G. A.	Univ. of Calif.
Almquist, H. J.	Emeryville, Calif.
Alt, Howard L.	Northwestern Univ. Med.
Althausen, T. L.	Univ. of Calif. Med.
Altschule, M. D.	Beth Israel Hosp., Boston
Altshuler, S. S.	Wayne Univ. Med.
Alvarez, Walter C.	Mayo Clinic
Alving, A. S.	Univ. of Chicago
Amberg, Samuel	Mayo Clinic
Amberson, W. R.	Univ. of Maryland Med.
Ambrose, A. M.	West Reg. Res. Lab., Albany, Calif.
Amoss, Harold L.	Rockefeller Inst.
Anderson, Dorothy H.	Coll. Phys. and Surg., N. Y.
Anderson, H. H.	Univ. of Calif. Med.
Anderson, John E.	Univ. of Minn.
Anderson, Richmond K.	Univ. of Buffalo
Anderson, Rudolph J.	Yale Univ.
Anderson, William E.	Rockville, Conn.
Andervont, H. B.	National Cancer Inst.
Andrus, E. C.	Johns Hopkins Univ.
Andrus, W. deW.	Cornell Univ. Med. Coll.
Angerer, C. A.	Ohio State Univ.
Angevine, D. M.	Wilmington, Del.
Ansbacher, Stefan	New York City
Antopol, William	Beth Israel Hosp., Newark, N. J.
Apperly, Frank L.	Med. Coll. of Va.
Aring, C. D.	Cincinnati Gen. Hosp.
Armstrong, Charles	Nat. Inst. of Health, Washington
Armstrong, W. D.	Univ. of Minn.
Arnold, Lloyd	Univ. of Ill.
Arnow, L. E.	Glenolden, Pa.
Aron, H. C. S.	Northwestern Univ. Med.
Aronson, J. D.	Henry Phipps Inst.
Asdell, S. A.	Cornell Univ.
Ashby, W. M.	Washington, D. C.
Asher, Leon	Berne, Switzerland
Ashman, Richard	Louisiana State Univ.
Asmundson, V. S.	Univ. of Calif.
Atchley, D. W.	Presbyterian Hosp., N.Y.C.
Aub, Joseph C.	Mass. Gen. Hosp., Boston
Auer, John	St. Louis Univ.
Austin, J. Harold	Univ. of Pa.
Avery, B. F.	Boston Univ.
Avery, O. T.	Rockefeller Inst., N. Y. C.
Avery, Roy C.	Vanderbilt Univ.
Aycock, W. L.	Harvard Med.
Ayo, C.	Montefiore Hosp., N. Y.

Babkin, Boris P.	McGill Univ.
Bachem, Albert	Univ. of Ill. Med. Coll.
Baehr, George	Mt. Sinai Hosp., N.Y.C.
Baernstein, H. D.	National Inst. of Health
Bagg, Halsey J.	Yorktown Heights, N. Y.
Bahrs, Alice M.	Portland, Ore.
Bailey, Cameron V.	N. Y. Post-Graduate Med.
Bailey, Percival	Univ. of Illinois Med.
Baitsell, George A.	Yale Univ.
Bakwin, Harry	N. Y. Univ. Med. Coll.
Baldwin, Francis M.	Univ. of S. Calif.
Baldwin, I. L.	Univ. of Wisconsin
Ball, G. H.	Univ. of Calif., L. A.
Ball, H. A.	San Diego, Calif.
Balls, A. K.	West. Reg. Res. Lab., Albany, Calif.
Barach, Alvan L.	Coll. Phys. and Surg., N. Y.
Barber, W. Howard	New York Univ. Med.
Bard, Philip	Johns Hopkins Univ.
Barer, Adelaide P.	State Univ. of Iowa
Barker, S. B.	Univ. of Iowa
Barlow, O. W.	Rensselaer, N. Y.
Barnes, R. H.	Glenolden, Pa.
Barnett, George D.	Stanford Univ.
Barr, David P.	Cornell Med. Coll.
Barron, E. S. G.	Univ. of Chicago
Barth, L. G.	Columbia Univ.
Bartley, S. H.	Dartmouth Coll.
Bass, Charles	Tulane Univ.
Bast, T. H.	Univ. of Wisconsin
Bates, R. W.	New Brunswick, N. J.
Batterman, R. C.	New York Univ. Med.
Bauer, J. H.	Rockefeller Inst.
Bauman, Louis	Presbyterian Hosp., N.Y.C.

Baumann, Carl A.	Univ. of Wisconsin
Baumann, E. J.	Montefiore Hosp., N.Y.C.
Baumberger, J. Percy	Stanford Univ.
Bayne-Jones, S.	Yale Univ.
Bazett, H. C.	Univ. of Pa.
Bean, John W.	Univ. of Mich.
Beard, J. W.	Duke Univ.
Beck, Claude S.	Western Reserve Univ.
Becker, E. R.	Iowa State Coll.
Beckman, Harry	Marquette Univ. Med.
Beckwith, T. D.	Univ. of Calif., L. A.
Beeson, P. B.	Emory Univ.
Behre, Jeannette A.	New York City
Belding, David L.	Boston Univ.
Bender, M. B.	Mt. Sinai Hosp., N. Y.
Bengston, Ida A.	National Inst. of Health, Washington
Bennett, G. A.	Univ. Illinois Med.
Bennett, L. L.	Univ. of Calif.
Berg, B. N.	Columbia Univ.
Berg, C. P.	State Univ. of Iowa
Berg, William N.	New York City
Bergeim, Olaf	Univ. of Ill.
Bergmann, Max	Rockefeller Inst.
Bernhard, Adolph	Lenox Hill Hosp., N.Y.C.
Bernhart, F. W.	Chagrin Falls, O.
Bernthal, T. G.	Vanderbilt Univ.
Berry, George P.	Univ. of Rochester Med.
Best, C. H.	Univ. of Toronto
Beutner, R.	Hahnemann Med. Coll., Philadelphia
Beyer, K. H., Jr.	Glenolden, Pa.
Bierman, W.	Mt. Sinai Hosp., N. Y.
Bieter, Raymond N.	Univ. of Minn.
Bills, C. E.	Mead, Johnson and Co., Evansville, Ind.
Bing, Franklin C.	Am. Med. Assn., Chicago
Bing, R. J.	Johns Hopkins Univ.
Birkhaug, Konrad E.	Geofysisk Inst., Bergen, Norway
Birnbaum, G. L.	New York Med. Coll.
Bishop, George H.	Webster Groves, Mo.
Biskind, G. R.	Mt. Zion Hosp., San Francisco
Blair, John E.	Hosp. for Joint Diseases, N.Y.
Blake, F. G.	Yale Univ.
Blalock, Alfred	Johns Hopkins Univ. Med.
Blatherwick, Norman R.	Metropolitan Life Ins. Co., N. Y. City
Blinks, L. R.	Stanford Univ.
Bloch, Robert G.	Univ. of Chicago
Block, Richard J.	N. Y. Med. Coll.
Block, Walter D.	Univ. of Michigan
Bloom, William	Univ. of Chicago
Bloomfield, A. L.	Stanford Univ. Med.
Bloor, W. R.	Univ. of Rochester
Blount, R. F.	Univ. of Texas Med.
Blum, Harold F.	National Cancer Inst.
Blumberg, Harold	Rensselaer, N. Y.
Blumgart, H. L.	Beth Israel Hosp., Boston
Bodansky, A.	Hosp. for Joint Diseases, N.Y.
Bodansky, Oscar	Edgewood Arsenal, Md.
Bodine, J. H.	State Univ. of Iowa
Bogen, Emil	Olive View, Calif.
Boissevain, Charles H.	Colorado Coll.
Bollman, Jesse L.	Mayo Clinic
Booher, Lela E.	Minneapolis, Minn.
Boor, Alden K.	Univ. of Chicago
Boothby, Walter M.	Mayo Clinic, Rochester, Minn.
Boots, Ralph H.	Presbyterian Hosp., N. Y.
Borsook, Henry	Calif. Inst. of Technology
Bornstein, S.	Jewish Hosp., Brooklyn
Bowen, B. D.	Buffalo Gen. Hosp.
Boyce, F. F.	New Orleans, La.
Boyd, Eldon M.	Queens Univ., Canada
Boyd, Theo. E.	Loyola Univ.
Boyden, E. A.	Univ. of Minn. Med.
Bozler, Emil	Ohio State Univ.
Bradford, William L.	Univ. of Rochester
Bradley, H. C.	Univ. of Wisconsin
Brand, Erwin	Coll. Phys. and Surg., N. Y.
Branham, Sara E.	Nat. Inst. of Health
Braun-Menendez, E.	Univ. of Buenos Aires
Brazda, F. G.	Louisiana State Univ.
Brewer, George	Univ. of Pa.
Briggs, A. P.	Univ. of Georgia
Brinkhous, K. M.	Univ. of Iowa
Brodie, B. B.	Goldwater Mem. Hosp., N. Y.
Broh-Kahn, R. H.	May Inst. Med. Research Cincinnati
Bronfenbrenner, J.	Washington Univ.
Bronk, D. W.	Univ. of Penn.
Brooks, Clyde	Essex Coll. of Med.
Brooks, C. M.	Johns Hopkins Univ.
Brooks, Matilda M.	Univ. of Calif.
Brooks, S. C.	Univ. of Calif.
Broun, G. O.	St. Louis Univ. Med.
Brown, Frank A., Jr.	Northwestern Univ.
Brown, J. Howard	Johns Hopkins Univ.
Brown, John B	Ohio State Univ.
Brown, Rachel	N. Y. State Dept. of Health
Browne, J. S. L.	Royal Victoria Hosp., Montreal
Bruger, Maurice	N. Y. Post-Graduate Med.
Brunschwig, Alexander	Univ. of Chicago
Buchanan, A. R.	Univ. of Colo. Med.
Buchanan, Robert E.	Iowa State Coll.
Buchbinder, W. C.	Michael Reese Hosp., Chicago
Bucy, Paul C.	Univ. of Illinois Med.

Bueding, Ernest	Western Reserve Univ.
Buell, Mary V.	Johns Hopkins Univ.
Bukantz, S. C.	Army Medical School
Bulger, H. A.	Washington Univ.
Bunney, W. E.	New Brunswick, N. J.
Burch, George E.	New Orleans, La.
Burch, John C.	Vanderbilt Univ. Med.
Burdon, Kenneth L.	Baylor Univ. Med.
Burk, Dean	National Cancer Inst.
Burky, Earl L.	Johns Hopkins Hosp.
Burn, C. G.	Long Island Coll. of Med.
Burns, E. L.	Louisiana State Univ.
Burns, Robert K., Jr.	Carnegie Inst., Baltimore, Md.
Burr, George O.	Univ. of Minn.
Burrows, M. T.	Pasadena, Calif.
Burrows, William	Univ. of Chicago
Burstein, C. L.	N. Y. Univ. Med.
Butcher, E. O.	N. Y. Univ. Dental Coll.
Butler, E. G.	Princeton Univ.
Butt, E. M.	Univ. of So. Calif.
Butts, Joseph S.	Oregon State Coll.
Byerly, T. C.	U. S. Animal Exp. Farm, Beltsville, Md.

Cahill, W. M.	Wayne Univ.
Calvery, Herbert O.	Food & Drug Admin.
Calvin, D. Bailey	Univ. of Texas Med.
Cameron, A. T.	Univ. of Manitoba
Cameron, John A.	Dallas, Texas
Campos, F. A. deM.	Univ. of Sao Paulo
Cannan, Robert K.	N. Y. Univ.
Cannon, Paul R.	Univ. of Chicago
Cantarow, Abraham	Jefferson Med. Coll.
Carey, E. J.	Marquette Univ.
Carlson, A. J.	Univ. of Chicago
Carmichael, E. B.	Univ. of Alabama Med.
Carmichael, L.	Tufts Coll.
Carpenter, C. M.	Univ. of Rochester
Carr, C. J.	Univ. of Md.
Carr, J. L.	Univ. of Calif. Hosp.
Cartland, G. F.	Kalamazoo, Mich.
Carruthers, A.	Birmingham, England
Cary, C. A.	U. S. Dept. of Agriculture
Casals, Jorge	Rockefeller Inst.
Casey, Albert E.	Birmingham, Ala.
Casida, L. E.	Univ. of Wisconsin
Castaneda, M. R.	Hosp. General, Mexico City
Cattell, McKeen	Cornell Univ. Med. Coll.
Cecil, R. L.	Cornell Univ. Med. Coll.
Cerecedo, L. R.	Fordham Univ.
Chadwick, C. S.	Vanderbilt Univ.
Chaikoff, I. L.	Univ. of Calif.
Chambers, Robert	New York Univ.
Chambers, Wm. H.	Cornell Univ. Med. Coll.

Chang, Hsi Chun	Peiping Union Med. Coll.
Chang, Hsiao-Chien	Hunan, China
Chargaff, Erwin	Coll. of Phys. and Surg.
Charipper, H. A.	New York Univ.
Chase, M. W.	Rockefeller Inst.
Cheer, S. N.	W. China Union Univ.
Chen, Graham M.	Univ. of Chicago
Chen, K. K.	Eli Lilly and Co., Indianapolis
Chen, T. T.	Peiping Union Med. Coll.
Cheney, R. H.	Long Island Univ.
Chidester, F. E.	Newark Valley, N. Y.
Child, C. M.	Stanford Univ.
Chouke, K. S.	Univ. of Pa.
Chow, B. F.	New Brunswick, N. J.
Christensen, K.	St. Louis Univ.
Christian, Henry A.	Harvard Univ.
Christman, Adam A.	Univ. of Mich.
Chu, F. T.	Peiping Union Med. Coll.
Chung, H. L.	Peiping Union Med. Coll.
Clark, Ada R.	Coll. of Phys. and Surg.
Clark, George	Orange Park, Fla.
Clark, Guy W.	Lederle Lab., Pearl River, N.Y.
Clark, P. F.	Univ. of Wisconsin
Clark, W. G.	Univ. of S. Calif.
Clarke, Hans T.	Coll. of Phys. and Surg.
Claude A.	Rockefeller Inst.
Clausen, H. J.	Univ. of Colo. Med.
Claussen, S. W.	Strong Memorial Hosp., Rochester, N. Y.
Clawson, Benjamin J.	Univ. of Minn.
Clifton, Charles E.	Stanford Univ.
Climenko, D. R.	Rensselaer, N. Y.
Clowes, G. H. A.	Eli Lilly & Co., Indianapolis
Code, C. F.	Mayo Foundation
Coggeshall, L. T.	Univ. of Mich.
Cohen, Barnett	Johns Hopkins Med.
Cohen, Milton B.	St. Alexis Hosp., Cleveland
Cohn, A. E.	Rockefeller Inst., N. Y.
Cohn, David J.	Michael Reese Hosp., Chicago
Cohn, Isidore	New Orleans, La.
Cole, Arthur G.	Univ. of Ill. Med.
Cole, Harold H.	Univ. of Calif., Davis
Cole, L. J.	Univ. of Wisconsin
Cole, Rufus I.	Rockefeller Inst., N. Y. City
Cole, Warren H.	Univ. of Ill. Med.
Cole, William H.	Rutgers Univ.
Colien, F. E.	Creighton Univ.
Collens, William S.	Brooklyn, N. Y.
Collier, William D.	St. Elizabeth's Hosp., Youngstown, O.
Collins, D. A.	Univ. of Illinois Med.
Collip, J. B.	McGill Univ.
Compere, E. L.	Univ. of Chicago
Conant, N. F.	Duke Univ.
Cook, Charles A.	St. Louis, Mo.

Cook, Donald H.	School of Tropical Med., San Juan, P. R.
Cook, E. S.	Cincinnati, O.
Cooke, J. V.	Washington Univ.
Cooper, Frank B	Chicago, Ill.
Cooper, Merlin L.	Univ. of Cincinnati
Cope, O. M.	N. Y. Med. Coll.
Copenhaver, W. M.	Columbia Univ.
Corbin, Kendall B.	Univ. of Tenn.
Corey, E. L.	Univ. of Va.
Cori, Carl F.	Washington Univ.
Corley, Ralph C.	Purdue Univ.
Corner, George W.	Carnegie Inst., Baltimore
Corper, H. J.	Nat. Jewish Hosp., Denver, Col.
Co Tui	N. Y. Univ. Med. Coll.
Coulson, E. J.	U. S. Dept. of Agriculture
Cowdry, E. V.	Washington Univ.
Cowgill, George R.	Yale Univ.
Cox, A. J., Jr.	Stanford Univ. Med.
Cox, Herald R.	Pearl River, N. Y.
Cox, Warren M., Jr.,	Mead Johnson Co.
Cram, Eloise B.	Nat. Inst. of Health
Crampton, C. Ward	N. Y. Post-Graduate Med.
Crandall, L. A., Jr.	Univ. of Tenn.
Creaser, C. W.	Wayne Univ. Med.
Crittenden, Phoebe J.	Westfield, N. J.
Crohn, Burrell B.	Mt. Sinai Hosp., N. Y.
Crossley, M. L.	Bound Brook, N. J.
Croxatto, H.	Catholic Univ., Chile
Cruz, W. O.	Rio de Janeiro, Brazil
Cruz-Coke, E.	Univ. of Santiago, Chile
Csonka, F. A.	U. S. Dept. of Agriculture
Culler, E. A.	Univ. of Rochester
Cummins, Harold	Tulane Univ.
Curtis, G. M.	Ohio State Univ.
Curtis, Maynie R.	Mason, Mich.
Cutler, Elliott C.	Peter Bent Brigham Hosp., Boston
Cutting, R. A.	Georgetown Univ. Med.
Cutting, W. C.	Stanford Univ. Med.
Cutuly, Eugene	Wayne Univ. Med.
Cuyler, W. K.	Duke Univ.
Dack, Gail M.	Univ. of Chicago
Daft, F. S.	Nat. Inst. of Health
Dakin, H. D.	Ossining, N. Y.
Dalldorf, Gilbert	N. Y. State Dept. of Health, Albany
Dalton, A. J.	National Cancer Inst.
Dam, Henrik	Univ. of Rochester Med.
D'Amour, F. E.	Univ. of Denver
Danforth, Charles H.	Stanford Univ.
Danforth, D. N.	Sloane Hosp., N. Y. City
Daniels, Amy L.	Iowa City, Iowa
Danzer, Charles S.	N. Y. Med. Coll.
Darby, E. M. K.	Naval Med. Res. Inst., Bethesda, Md.
Dautrebande, Lucien	Univ. of Liege, Belgium
Davenport, C. B.	Sta. for Exp. Evolution, Cold Spring Harbor, N. Y.
Davidsohn, Israel	Univ. of Chicago
Davis, H. A.	Louisiana State Univ.
Davis, J. E.	Univ. of Arkansas
Davis, M. E.	Univ. of Chicago
Dawson, James A.	Coll. City of New York
Day, A. A.	Northwestern Univ. Med.
Day, Harry G.	Indiana Univ.
Day, Paul L.	Univ. of Arkansas Med.
DeBakey, M. E.	Tulane Univ.
DeBodo, Richard	New York Univ. Med.
Decherd, George M.	Univ. of Texas Med.
DeEds, Floyd	U. S. Dept. of Agriculture
DeFries, R. D.	Univ. of Toronto
DeGara, P. F.	Cornell Univ. Med.
DeGowin, E. L.	State Univ. of Iowa
DeGraff, A. C.	N. Y. Univ. Med.
del Castillo, E. B.	Univ. of Buenos Aires
Dennis, E. W.	Amer. Univ. of Beirut
DeRenyi, G. S.	Univ. of Pa.
De Savitsch, Eugene	Washington, D. C.
Detwiler, S. R.	Columbia Univ.
Deuel, Harry J., Jr.	Univ. of S. Calif. Med.
Deulofen, V.	Univ. of Buenos Aires
Dick, George F.	Univ. of Chicago
Dieckmann, W. J.	Univ. of Chicago
Dienes, Louis	Mass. Gen. Hosp., Boston
Dieuaide, Francis R.	Harvard Univ.
Dille, J. M.	Univ. of Illinois
Dingle, J. H.	Boston City Hosp.
Doan, Charles A.	Ohio State Univ.
Dochez, A. R.	Presbyterian Hosp., N. Y. C.
Dock, William	New York City
Dohan, F. C.	Univ. of Pa.
Doisy, Edward A.	St. Louis Univ.
Dolley, W. L., Jr.	Univ. of Buffalo
Dominguez, R.	St. Luke's Hosp., Cleveland
Domm, L. V.	Univ. of Chicago
Donahue, J. K.	Coll. of Charleston, S. C.
Donaldson, J. C.	Univ. of Pittsburgh
Dorfman, A.	Univ. of Chicago
Dorfman, Ralph I.	Western Reserve Univ.
Dotti, L. B.	St. Luke's Hosp., N. Y. City
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